1987

FURTHER STUDIES ON THE IMMUNOLOGY OF THE ALPHA TOXIN OF STAPHYLOCOCCUS AUREUS.

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FURTHER STUDIES ON THE IMMUNOLOGY OF THE ALPHA TOXIN OF *STAPHYLOCOCCUS AUREUS*

by
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A dissertation
submitted to the faculty of Graduate Studies
through the
Department of Biological Sciences
in partial fulfillment for the degree of
Doctor of Philosophy
at the
University of Windsor

Windsor, Ontario.
Canada
1987

ISBN 0-315-39637-7

ABSTRACT

A double antibody sandwich enzyme- linked immunosorbent assay (ELISA) was developed for measuring *Staphylococcus aureus* alpha toxin. This assay was 500 to 1, 000 times more sensitive than the commonly used hemolytic titration assay, and was less variable. The binding of alpha toxin to the adsorbed antibody was most effective after an overnight incubation at 27 C. The toxin was detectable even at a $\log_2$ 17 dilution of an *S. aureus* culture supernatant.

A modified version of the ELISA, a competitive enzyme- linked immunosorbent assay (CELIA) was developed for detection of immunologically active fragments of alpha toxin. In the CELIA, immunologically reactive fragments bound to the adsorbed antitoxin. However, since these fragments were unable to simultaneously bind the second (enzyme- labelled) antibody, a decrease in absorbance was noted in these wells.

Fragmentation analysis of alpha toxin with trypsin, under a variety of conditions, produced a variety of fragments which were recognized by either antibinding, or indirect hemagglutinating antibodies. However, the most striking feature of these results, was a 20, 000 Dalton fragment that was recognized by both populations of antibodies. This fragment appeared early in the digestion course and was resistant to further tryptic digestion. A similarly sized fragment also spontaneously appeared in purified alpha toxin preparations.

Cyanogen bromide digestion of alpha toxin produced five fragments that were recognized by rabbit antitoxin; the molecular weights of these fragments ranged from 9, 000 to 12, 000 Daltons. The 9, 000; 10, 000 and 12, 000 Dalton fragments reacted with antibinding antibodies. The 9, 000; 10, 000; 10, 500 and 11, 000 Dalton fragments reacted with indirect hemagglutinating antibodies. The 9, 000 Dalton

fragment also contained the site that binds to the rabbit erythrocyte receptor.

Five monoclonal antibodies (MAb- 1 to MAb- 5) to alpha toxin were developed. However, when the relative specificities of these antibodies were examined by ELISA, MAb- 1 and MAb- 2 recognized either the same determinant, or, two determinants that are close together. Monoclonal antibodies 1, 3, 4, and 5, recognized different antigenic determinants. All the monoclonal antibodies protected rabbit erythrocytes from alpha toxin- mediated lysis. When examined in the IHA test MAbs- 1, 2, 4, and 5 agglutinated toxoid- coated erythrocytes. This indicated that these antibodies recognized determinants that were outside of the erythrocyte receptor binding site of alpha toxin. Monoclonal antibody- 3, an antibinding antibody, also did not bind to the 9, 000 Dalton fragment that contains this binding site. Monoclonal antibody- 3 therefore recognizes an antigenic determinant that is outside of the binding site, but, is still close enough so that the bound antibody molecule sterically hinders simultaneous binding to the receptor.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. H.B. Fackrell for his guidance and support over the years. I am grateful to Dr. D.A. Cotter, Dr. A.H. Warner and Dr. K.E. Taylor for serving on my doctoral committee. I would also like to thank my external examiner, Dr. S. Harshman. Many thanks to Ms. J. Dosescu for excellent technical assistance in the hybridoma laboratory. Finally I would like to thank the fellow students in my laboratory, both past and present.

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LIST OF ABBREVIATIONS

AB–Ab antibinding antibody
C degrees centigrade
CELIA competitive enzyme–linked immunosorbent assay
CNBr cyanogen bromide
ELISA enzyme–linked immunosorbent assay
g unit of gravity
HAT Hypoxanthine Aminopterin Thymidine
HPLC High Performance Liquid Chromatography
HU hemolytic unit of alpha toxin
IgG immunoglobulin G
IHA indirect hemagglutinating
IHA–Ab indirect hemagglutinating antibody
MAb Monoclonal antibody
OPD o–phenylenediamine
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffered saline
PNP p–nitrophenol
PNPP p–nitrophenyl phosphate
RIA radioimmunoassay
rpm revolutions per minute
SDS sodium dodecyl sulfate
TBS Tris buffered saline
TEMED N,N,N',N'–tetramethylethylene diamine

INTRODUCTION

The Gram positive organism, *Staphylococcus aureus*, produces a large number of extracellular products which includes enzymes and a variety of toxins (Kloos and Schleifer, 1986). The most potent of these products is considered to be the alpha toxin (Arbuthnott, 1970). This toxin is cytotoxic for a wide variety of cells, lyses erythrocytes, is dermonecrotizing, and is lethal for a number of laboratory animals. Intravenously injected radiolabelled alpha toxin has been found widely distributed in the body (Jeljaszewicz *et al.*, 1969) but, the *in vivo* target organ has not been identified. However, there is general agreement that the cell membrane constitutes the primary target site of the toxin.

The majority of studies on this toxin have utilized the rabbit erythrocyte which is the most sensitive type of cell. A rabbit erythrocyte receptor for this toxin has been identified as the carbohydrate moiety (Simpson, 1986, MSc. thesis) of band three (Maharaj and Fackrell, 1980). The unique susceptibility of the rabbit erythrocyte has made this cell a convenient vehicle for the measurement of alpha toxin. The hemolytic titration assay measures the amount of hemoglobin released as a result of alpha toxin- mediated lysis of the erythrocytes. There are associated with this assay however, several inherent problems such as variability and lack of standardization which limit its use as a quantitative method.

The exact role of alpha toxin in the pathogenesis of staphylococcal infections and the protective role of anti- alpha toxin antibodies is not clear. These problems may not be resolved until the toxin is antigenically characterized, and its mode of action understood. Studies have shown that the toxin is composed of a single polypeptide chain of molecular weight 10, 000 to 45, 000 Daltons (Freer and Arbuthnott, 1983; Wiseman, 1975). The amino acid composition of this toxin has been examined by several groups and their results are in reasonable agreement

(Bernheimer and Schwartz, 1963; Coulter, 1966; Fackrell and Wiseman, 1976a; Six and Harshman, 1973a). In addition, the alpha toxin gene has been sequenced (Gray and Kehoe, 1984) and the amino acid composition that was predicted from the gene sequence corresponds well with the protein derived data. Several groups have performed structure– function studies on alpha toxin using enzyme or cyanogen bromide generated fragments (Blomqvist and Thelestam, 1986a; Harshman *et al*, 1986; Kato and Watanabe, 1980; Watanabe and Kato, 1978). From the results of these studies, it is becoming recognized that discrete regions on the toxin might be responsible for the various biological properties that are displayed by this molecule. However, of the structure– function studies performed so far, only one (Harshman *et al*, 1986) has utilized an anti- alpha toxin antibody as the investigative tool. Previously, it was demonstrated that at least two populations of anti - alpha toxin antibodies exist. One population prevents the toxin from binding to the erythrocyte receptor. The other population neutralizes toxin that is already bound to the receptor. Thus the use of these antibodies as molecular probes should pinpoint regions of the toxin that are responsible for binding and membrane damage. Furthermore, the manufacture and identification of a greater variety of antitoxins by use of monoclonal antibody technology should also facilitate the identification of additional regions on the toxin that may be responsible for the other biological activities that are displayed by this molecule.

This study was designed to continue the structure - function analysis of alpha toxin. The three main objectives that were addressed are as follows:

(1) The development of an assay for alpha toxin that abrogates the problems that are associated with the hemolytic titration assay. The assay chosen was a double antibody sandwich ELISA which matches the great sensitivity of the RIA but

does not involve the health hazards that are associated with it.

(2) Fragmentation of the alpha toxin molecule by use of proteases and cyanogen bromide. Fragments of the molecule are more amenable to structure– function analysis. This approach combined with an SDS PAGE/ immuno electrotransfer blot system which utilized antitoxin as probes, was considered to be a feasible method for the isolation and identification of regions of the toxin which might be of biological interest.

(3) The development of monoclonal antibodies directed to determinants that are located in different regions of the molecule. Use of these antibodies will complement the structure– function studies performed with the polyclonal antibodies.

LITERATURE REVIEW

Discovery

The relationship between pathogenicity of bacteria and toxin production was postulated since 1872 (Klebs, 1872). Around the turn of the century, the production of toxins by the staphylococci was investigated by several investigators (Brieger and Fraenkel, 1890; de Christmas, 1888; Kraus and Clairmont, 1900; Kraus and Pribram, 1906; Leber, 1888; Neisser and Wechsberg, 1901; Rodet and Courmont, 1892; Van de Velde, 1894). These researchers noted that broth cultures of staphylococci displayed toxic manifestations such as hemolysis, dermonecrosis and lethality for some animals. In 1928, the Bundaberg disaster (Elek, 1950) focussed increased attention on the toxic and invasive properties of staphylococci. The fluid obtained from a *Staphylococcus aureus* infected preparation of diptheria toxoid was hemolytic and lethal for rabbits. Burnet (1929; 1931) showed that antisera from rabbits injected with crude bacterial filtrate abolished all of the three toxic manifestations. In view of this, Burnet (1931) suggested that one toxin was responsible for the three characteristic activities of staphylococcal filtrates.

Since then, it has been shown that *Staphylococcus aureus* produces four types of hemolysins. These are termed alpha, beta, gamma and delta, in the order of their discovery. Although all of these hemolysins lyse erythrocytes and are cytotoxic to a variety of mammalian cells, the specificity and mode of action of each is distinctive. The alpha hemolysin possesses hemolytic, dermonecrotic and lethal activities (Bernheimer and Schwartz, 1963; Goshi *et al.*, 1963; Jackson, 1963; Lominski *et al.*, 1963; Kumar and Lindorfer, 1962; Kumar *et al.*, 1962). Rabbit erythrocytes have been found to be more sensitive to this hemolysin than erythrocytes of any other species. A receptor for alpha toxin has been identified on the rabbit erythrocyte membrane (Maharaj and Fackrell, 1980; Simpson, 1986).

The primary site of action of this toxin seems to be the cell membrane. However, the *in vivo* target organ has not been identified and the mode of action is not yet understood.

Walbum (1921) and Bigger *et al*, (1927) described a "hot– cold" phenomenom. Incubation of dilutions of an undefined staphylococcal hemolysin in the presence of sensitive erythrocytes at 37 C followed by refrigeration at 4 C caused intensification of hemolysis. This hemolysin was subsequently found to be relatively active on sheep erythrocytes (Bigger, 1933). This finding was reiterated by Glenny and Stephens (1935) who described a hemolysin that was serologically distinct from alpha hemolysin. This hemolysin, termed beta hemolysin, contrasted with the alpha hemolysin in being hemolytic for sheep erythrocytes but not rabbit erythrocytes; non– dermonecrotizing in guinea pig skin and non– lethal for mice. Beta hemolysin has been demonstrated by Doery *et al*, (1963, 1965) Maheswaran *et al*, (1967) Wadstrom and Mollby (1971a, b) and Wiseman and Caird (1967) to be a Mg^{2+} dependent phospholipase C that is specific for sphingomyelin.

Morgan and Graydon (1936) reported an alpha hemolysin preparation that consisted of two distinct lytic substances. These were termed alpha 1 and alpha 2 and were found to be antigenically different. Smith and Price (1938) proposed the existence of gamma hemolysin. Antisera selected to show a high gamma/ alpha ratio also had a high alpha 2/ alpha 1 ratio. This suggested that gamma hemolysin might be identical with the alpha 2 hemolysin of Morgan and Graydon (1936). Elek and Levy (1950) found that the characteristic patterns caused by the hemolysins on blood agar were consistent with the existence of only three hemolysins; alpha, beta and delta. These researchers decided that the alpha 2 , gamma and delta hemolysins were identical. However, it was subsequently shown that their method could not detect gamma hemolysin since it is inhibited by agar. Marks (1951)

agreed that alpha 2 and delta hemolysins were identical but argued for the separate existence of gamma hemolysin. Gamma hemolysin remained virtually undefined until several studies gave details for its production and purification in workable yields from strain Smith 5R (Bezard and Plommet, 1973; Guyonnet and Plommet, 1970; Guyonnet *et al*, 1968 and Plommet and Bouillame, 1966). Gamma hemolysin is hemolytic towards human, rabbit and sheep erythrocytes (Fackrell, 1974; Guyonnet and Plommet, 1970). Taylor and Bernheimer (1974) showed that gamma hemolysin is inhibited by several lipids and sterols in which the 3– B– hydroxy group is esterified. Gamma hemolysin is also inhibited by cardiolypin, phosphatidyl inositol, phosphatidyl choline, phosphatidyl glycerol, phosphatidyl serine, nervonic acid, stearic acid, and palmitic acid all at concentrations of 0.05 – 0.5 ug/ ml. No change in erythrocyte lipids has been observed after exposure to gamma hemolysin. The mode of action of this hemolysin remains unknown. Fackrell and Wiseman (1976b) demonstrated a release of nitrogen and acid soluble phosphorous from gamma hemolysin treated human erythrocytes. This suggests that gamma hemolysin possesses phospholipase activity.

In 1947, Williams and Harper detected delta hemolysin in strains of *Staphylococcus aureus* grown on sheep blood agar to which alpha and beta anti-hemolysins were added. Delta hemolysin does not show the species specificity demonstrated by both alpha and beta hemolysins (Marks and Vaughn, 1950; Kayser and Renaud, 1965). Instead, it has a wide spectrum of activity affecting most cell types that have been tested. Delta hemolysin from the Newmann and E– Delta strains of *Staphylococcus aureus* liberate organic phosphorous from erythrocytes in proportion to their hemolytic sensitivity (Wiseman and Caird, 1976). An actual substrate in the erythrocyte membrane has not been identified. Bernheimer (1974) has presented evidence that delta hemolysin acts via high

surfactant activity and hydrophobicity, apparently causing hemolysis by its detergent– like action. Studies of the interaction of delta hemolysin with phospholipid monolayers suggest that this hemolysin may aggregate in the plane of the membrane forming transmembrane channels that result in the loss of selective ion permeability (Bhakoo *et al.*, 1982). Freer and Birbeck (1982) suggested that delta hemolysin possesses an alpha helical conformation which would result in a rod shaped molecule with amphipathic properties. This conformation would allow delta hemolysin to orient itself across the bilayer.

Terminology

Alpha hemolysin is also referred to as alpha lysin (Freer and Arbuthnott, 1976; 1983). However, apart from its erythrocyte– lysing function, this protein also damages blood platelets (Seigel and Cohen, 1964) damages leucocytes (Gladstone, 1966; Jeljascewicz, 1972; McGee *et al.*, 1983) human diploid fibroblasts, HeLa cells, rabbit kidney cells (Artenstein *et al.*, 1963) adrenocortical tumor cells (Thelestam and Blomquist, 1984) and epithelial cells (fibroblasts) (Thelestam, 1983a). In addition, this protein causes contraction and paralysis of vascular smooth muscle (Thal and Enger, 1961) and spastic paralysis of mouse skeletal muscle (Lominski *et al.*, 1962). Nervous tissue is also affected by exposure to alpha hemolysin. Edelwejn *et al.*, (1976) demonstrated abnormal EEG recordings just prior to death in rabbits. Harshman *et al.*, (1981; 1985) Szmigielski and Harshman, (1978) and Szmigielski *et al.*, (1979) showed that alpha hemolysin preferentially injures the myelin sheaths of isolated nerves. Alpha hemolysin also adversely affects the circulatory system by causing a specific increase in vascular permeability (Jeljascewicz, 1972) and induces the release of prostacyclin (a potent vasodilator)

from epithelial cells (Suttorp *et al.*, 1985). Thus, it is clear that the term "hemolysin" fails to adequately describe the broad spectrum of this protein's biological activities. Bernheimer (1974) proposed instead, the term "cytolytic toxin", which he defined as a bacterial product capable of causing physical dissolution of a variety of cells *in vitro*. McCartney and Arbuthnott (1978) however, suggested the term "membrane– damaging toxins", since low concentrations of this protein is capable of causing permeability changes without actual cell lysis. Finally, in 1979, Rogolsky suggested that "the term hemolysin... be retired with due recognition of its past distinction". Currently, the term alpha toxin is in general use with a few exceptions.

Physiochemical Characteristics

Alpha toxin is a protein consisting of one polypeptide chain which is devoid of any disulphide bonds or sulfhydryl groups (Arbuthnott, 1970; Bernheimer and Schwartz, 1963; Lominski *et al.*, 1963; Six *et al.*, 1973a, b). One report that the toxin also contains carbohydrate (Goshi *et al.*, 1963) has never been substantiated. The toxin is positively charged and migrates towards the cathode (Coulter, 1966). Various reports, as summarized by Wiseman (1975) and Freer and Arbuthnott (1983) have shown the molecular weight of this toxin to be in the range of 10 kilodaltons to 45 kilodaltons. The variation probably reflects the methods of estimation used. Six and Harshman (1973b) using four different methods to assess a single batch of alpha toxin, reported the molecular weight to be in the range of 26 kilodaltons to 31 kilodaltons.

Several amino acid analyses have been performed on alpha toxin and the results are in reasonable agreement (Bernheimer and Schwartz, 1963; Coulter, 1966;

Fackrell and Wiseman, 1976a; Kato and Watanabe, 1980; Six and Harshman, 1973a; Watanabe and Kato, 1978; Wiseman and Caird, 1970) although the preparation of Fackrell and Wiseman (1976a) contained a higher concentration of proline, glycine and alanine than those of the other investigators. Different amino acids have been reported to be the N– terminus of alpha toxin. Coulter (1966) detected histidine and arginine as N– terminal amino acids. Six and Harshman (1973a, b) and Watanabe and Kato (1978) reported alanine to be in this position. Fackrell and Wiseman (1976a) and Wiseman and Caird (1970) reported histidine to be the N–terminus amino acid. The presence of proteases during the purification procedures (Dalen, 1976b) may explain both the variation in N– termini and molecular weights that have been reported for this toxin.

Sedimentation coefficient studies performed on alpha toxin have revealed the presence of multiple forms of this molecule. However, the major component of alpha toxin has a sedimentation coefficient of around 3S (Bernheimer and Schwartz, 1963; Cooper *et al.*, 1966; Coulter, 1966; Fussle *et al.*, 1981; Goode and Baldwin, 1973; Lominski *et al.*, 1963; Six and Harshman, 1973a, b). Fackrell and Wiseman (1976b) reported a sedimentation coefficient of 1.4S for freshly prepared alpha toxin. However, after three days in physiological buffer, the sedimentation coefficient value increased to 2.8S. Forlani *et al.*, (1971) reported a minor 2S component in their 2.8S preparation. In highly purified preparations of alpha toxin, a component has been found to have sedimentation coefficients between 10S to 16S. This component has been speculated to represent aggregated or polymerized forms of the toxin that are biologically inactive (Arbuthnott *et al.*, 1967). Arbuthnott *et al.*, (1967) further observed that 12S toxin disaggregated in urea to yield biologically active 3S toxin. Bernheimer (1974) noted that as much as 30 percent of purified 3S toxin may be in the polymerized 12S form. However, unlike

Arbuthnott *et al.*, (1967) Bernheimer (1974) found the 12S toxin to be sometimes hemolytic. Freer *et al.*, (1968) demonstrated that negatively stained 12S toxin consist of small rings 9nm to 10 nm in diameter (outer) which formed part of a hexagonal array of six subunits each 2 nm to 2.5 nm in diameter. In contrast, 3S toxin was shown to be amorphous. Dalen (1976c) also observed similar results. Wiseman (1975) reasoned that the "Arrhenius" effect that is demonstrated by alpha toxin may be the result of interconversion between the 12S and 3S forms of the molecule.

Sucrose density centrifugation performed by Bernheimer and Schwartz (1963) also yielded multiple forms of alpha toxin. Four electrophoretically distinct peaks were detected. However these peaks were all biologically similar. Different components of alpha toxin were also distinguished by isoelectric focussing. Several groups (Fackrell and Wiseman, 1976b; Goode and Baldwin, 1973, 1974; McNiven *et al.*, 1972; Wadstrom, 1968) found that alpha toxin could be separated into components of four different isoelectric points (pI). In these studies however, the main component of pI 8.5 accounted for over 80 percent of the recovered hemolytic activity, with the three additional minor peaks also being hemolytic. Wadstrom (1968) found that when each of the components were refocussed, the same pattern of four pIs were observed.

Production and purification

Over the years there have been numerous published schemes for the purification of alpha toxin. These methods, which have been reviewed extensively (Arbuthnott, 1970; Freer and Arbuthnott, 1983; Rogolsky, 1979; Wiseman, 1975) involve the use of many different kinds of protein purification methods.

Precipitation methods, gel filtration, electrophoresis, isoelectric focussing and ion exchange chromatography are most commonly used. A complex procedure involving zinc chloride precipitation, gel filtration, starch zone electrophoresis, ion exchange chromatography, Pevikon zone electrophoresis and dialysis against ammonium sulphate has been used by Watanabe and his co– workers. (Watanabe, 1976; Watanabe and Kato, 1974). On the other hand, Harshman and his colleagues have presented a more elegant method which features adsorption of alpha toxin onto controlled pore size glass beads as a concentration step followed by ion exchange chromatography (Cassidy and Harshman, 1976c). Another simplified procedure was presented by Dalen (1976a, b) who precipitated out the toxin from culture filtrate by heating at 60 C for 20 minutes. This inactivated the toxin and contaminating proteases. The toxin was reactivated in 8 molar urea and further purified by polyacrylamide electrophoresis at pH 8.6. A unique procedure was published by Fussle *et al.* (1981). This group took advantage of the fact that alpha toxin binds to rabbit erythrocyte membranes. Hypotonically lysed membranes were incubated with *Staphylococcus aureus* culture supernatant, washed, and the toxin dissolved in 1 percent Triton– X 100 and 1 percent deoxycholate. The detergent extract was then centrifuged and purified by Sepharose 6 B chromatography and a protein of 300– 700 kD eluted. This protein was shown by ultracentrifugation to correspond to the 12s complex of alpha toxin. The complex was broken down to 36 kD components by SDS gel electrophoresis and identified using specific antisera and by its typical appearance in the electron microscope.

Biosynthesis

The strain of *Staphylococcus aureus* most commonly used for studies on alpha toxin is Wood 46, although it is acknowledged that considerable variation in properties occur within this strain (Freer and Arbuthnott, 1983). However, as noted by Goode and Baldwin (1974) alpha toxin produced by different strains is very similar in biological and chemical properties.

Limited information is available about nutritional factors which influence the production of alpha toxin. Parker *et al.*, (1926) increased the yield of toxin by incubating the cultures under 10 percent CO_2 in air. Burnet (1930) found that 20 to 40 percent CO_2 and 0.3 percent agar enhanced alpha toxin production in his Wood 46 strain. The amino acids glycine, arginine and proline were found to be essential in toxin production (Gladstone, 1938). Dalen (1973c) confirmed the requirement for arginine and glycine, but also noted that serine and histidine increased yields. Dalen (1973a, b) further suggested that the stimulating effect of CO_2, serine and glycine, were related to their role as precursors of histidine. However, stimulation of alpha toxin production was not directly correlated with free intracellular histidine. Duncan and Cho (1972) have reported that production of alpha toxin is maximal at a glucose concentration of 0.2 percent.

Alpha toxin is produced in a biphasic manner in liquid batch cultures. Low level production occurs during exponential growth with a marked increase occuring just before the onset of a slower rate of growth prior to the stationary phase (Coleman and Abbas– Ali, 1977; Duncan and Cho, 1971). By stationary phase, alpha toxin can account for up to 2 percent of the total dry weight of the culture (Bernheimer and Schwartz, 1963: Duncan and Cho, 1971) and is localized on the inner surface of the cytoplasmic membrane prior to its release (Coulter and Mukherjee, 1971). It has been reported that alpha toxin is released from intact

cells (Bernheimer and Schwartz, 1963; Duncan and Cho, 1971). However there is now uncertainty about the authenticity of these data. Both Bernheimer and Schwartz (1963) and Duncan and Cho (1971) monitored the appearance of nucleic acid in the culture supernatant as a marker for autolysis. However, Freer and Arbuthnott (1983) reported that most of the high molecular weight DNA remains associated with the cells or remains intracellular in spite of extensive membrane disruption.

In a study of the transport and processing of alpha toxin, Tweten *et al.*, (1983) identified and partially characterized two larger precursor proteins of the toxin. Both of these precursor proteins were present in the cell membrane at very low levels and appeared to be rapidly processed to the mature form. The larger precursor protein contained four additional peptides and the smaller precursor protein contained three additional peptides not found in the extracellular toxin. These additional sequences were presumed to be due to signal peptides which are cleaved before or during secretion. Fairweather *et al.*, (1983) and Kehoe *et al.*, (1983) described the cloning and expression of the alpha toxin determinant in *Bacillus subtilis* and *Escherichia coli* K−12 respectively. It was found that in *Bacillus subtilis*, the alpha toxin was secreted by the cells as two polypeptides of 33 and 34 molecular weight. It was speculated that these two forms may represent different processed forms of the polypeptide with and without a signal sequence. In 1984, Gray and Kehoe reported the complete DNA sequence of a cloned alpha toxin gene. They found that the primary product of the cloned alpha toxin gene contained a 26 amino acid leader sequence which possessed characteristic features of a signal sequence that may be involved in secretion.

Considerable interest has been directed towards the examination of the genetic control of alpha toxin. Alpha toxin production is not ubiquitous among all strains

of *Staphylococcus aureus*, and the stability of toxin production varies among the strains that do (Elek, 1959). In some strains of *Staphylococcus aureus*, there appears to be a link between toxin production and lysogeny. Blair and Carr (1961) demonstrated that the ability to produce alpha toxin was often acquired by a non-toxigenic strain after lysogenization by a phage derived from a toxin producing strain. This finding conflicts with the failure of Hendricks and Alternbem (1968) to find evidence of a prophage in alpha toxin production. Witte (1976) found a high spontaneous rate of loss of the alpha toxin marker in clinical isolates of *Staphylococcus aureus* and concluded that in some strains, alpha toxin activity was controlled by a plasmid. In a genetic study of alpha toxin mutants of *Staphylococcus aureus*, McClatchy and Rosenblum (1966) found mutants that fell into two distinct genetic groups. Members of the first group showed pleiotropic effects on alpha toxin and fibrinolysin production and probably represented a regulatory gene locus for both proteins. The other group represented hemolysis-regulatory mutants which produced material cross- reacting with alpha toxin. This group probably represented mutants in the structural gene for alpha toxin. Recombination was observed only between mutants in the different groups. Wheeler (1975) and Brown and Pattee (1980) also observed a close association between the production of alpha toxin and fibrinolysin. Studies by Pattee *et al.*, (1975, 1978, 1980) indicated that there are three genetic linkage groups for the chromosome of *Staphylococcus aureus*, and that the gene (Hla^+) that controls alpha toxin production is in linkage group three. In strain 8325, the Hla^+ phenotype was always associated with fibrinolysin production, whereas the Hla^- phenotype was non- fibrinolytic. This confirmed the findings of McClatchy and Rosemblum (1966) and Wheeler (1975). From these findings, it seems possible that the Hla^+ gene, instead of being a structural gene for alpha toxin, plays an

undefined role in the regulation of several extracellular proteins of which alpha toxin is but one. Brown and Pattee (1980) also found a high degree of instability in the Hla^+ gene that is uncharacteristic of a conventional chromosomal gene. They suggested the possibility that the Hla^+ gene was on a transposable genetic element. This proposition which was also made by Rogolsky (1979) offers a tenable explanation for the high numbers of Hla^- derivatives of strain 8325 and the fact that Hla^- derivatives did not revert back to Hla^+. Such an element which may or may not be site specific (Phillips and Novick, 1979) would also offer an explanation for the earlier observations that alpha toxin production is under plasmid control in some strains and is observed in phage conversion in others. No evidence has yet been presented to substantiate this proposition. Evidence in support of the Hla^+ gene being responsible for alpha toxin production has recently been presented by two groups. Mallonee *et al.*, (1982) and Rescei *et al.*, (1986) reported that insertion of the transposon Tn 551 into the Hla chromosomal locus depressed production of alpha toxin. Rescei *et al.*, (1986) also found that this insertion of Tn 551 element elevated the production of another exoprotein, protein A. This coordinate control of alpha toxin and protein A in *Staphylococcus aureus*, was also observed by Janzon *et al.*, (1986). In both of these studies, the defect in the alpha toxin expression was at the transcriptional level. Rescei *et al.*, (1986) concluded that the insertion of the transposon inactivated a trans– active positive control element which they designated *agr* for accessory gene protein.

Biological activities

Staphylococcal alpha toxin is defined as having hemolytic, dermonecrotic, lethal, platelet damaging and cytotoxic activities (Freer and Arbuthnott, 1976; 1983; Jeljaszewicz 1972; Jeljaszewicz *et al.*, 1978; Rogolsky, 1979; Wiseman, 1975). Apart

from its erythrocyte– lysing functions, this protein also damages blood platelets (Seigel and Cohen, 1964) leucocytes (Gladstone, 1966; Jeljascewicz, 1972; Maheswaran *et al.*, 1969; McGee *et al.*, 1983) human diploid fibroblasts, HeLa cells, rabbit kidney cells (Artenstein *et al.*, 1963) adrenocortical tumor cells (Thelestam and Blomquist, 1984) and epithelial cells (fibroblasts) (Thelestam, 1983a). In addition, alpha toxin causes contraction and paralysis of vascular smooth muscle (Thal and Egner, 1961) and spastic paralysis of mouse skeletal muscle, both *in vivo* and *in vitro* (Lominski *et al.*, 1962). Nervous tissue is also affected by exposure to alpha toxin. Edelwejn *et al.*, (1976) demonstrated alpha toxin– induced abnormal EEG recordings just prior to death in rabbits. Harshman *et al.*, (1981; 1985) Szmigielski and Harshman (1978) and Szmigielski *et al* (1979) showed that alpha toxin preferentially injures the myelin sheaths of isolated nerves. The level of toxin used was such that no effect on electrical potential or ion leakage was noticed, but extensive disruption of the myelin occured. Alpha toxin also adversely affects the circulatory system by causing a specific increase in vascular permeability (Jeljascewicz, 1972) and induces the release of prostacyclin (a potent vasodilator) from epithelial cells (Suttorp *et al.*, 1985).

Work is now in progress to determine if the various biological activities exhibited by this protein, can be localized in domains on the molecule. Studies have suggested that alpha toxin has separate regions responsible for receptor binding and for membrane damage to RRBC (Cassidy and Harshman, 1976a, b; Barei and Fackrell, 1979) and to adrenocortical tumor cells (Thelestam and Blomquist, 1984). Watanabe and Kato (1978) and Kato and Watanabe (1980) have described two tryptic fragments each of which contained hemolytic or lethal activities, but not both. Harshman *et al.*, (1986) have performed a structure function analysis using CNBr fragments (Six and Harshman, 1973b) of alpha toxin

and have suggested that the C– terminus segment does not interfere with binding to the specific receptor or with the formation of hexamers. Blomqvist and Thelestam (1986a) have obtained an 18.5 kiloDalton fragment of the toxin, which is hemolytic but not membrane– damaging, or lethal. Furthermore, this fragment possesses the region responsible for binding to a target cell. Based on their findings this group have presented a hypothetical map of the different biologically active regions of alpha toxin.

The large diversity of cell types that are affected by this toxin suggest that susceptible cells possess a common target site for the alpha toxin molecule. Since the cell membrane constitutes the primary contact site with the toxin, studies have focussed attention on the interaction between the toxin and membranes. These studies include methods involving fluorescein labelled antitoxin (Kaplan,1972) fluorescein labelled anti– immunoglobulin (Klainer *et al.*, 1964) ^{125}I monoiodinated alpha toxin (Cassidy and Harshman, 1973, 1976) fluorescein labelled toxoid (Barei and Fackrell, 1979) adsorption of toxin with membranes (Wiseman *et al.*, 1975) electron microscopy (Bernheimer *et al.*, 1972; Freer *et al.*, 1968, 1973) release of ^{35}S methionine from pre– labelled rabbit kidney and human ammnion cells after treatment with alpha toxin (Artenstein *et al.*, 1963) leakage of different sized radio– labelled markers from toxin treated human lung fibroblasts (Thelestam and Mollby, 1976) structural damage to human lung fibroblasts by alpha toxin (Thelestam *et al.*, 1973; Thelestam and Mollby, 1973b) and SDS– PAGE immunoblotting (Bhakdi *et al.*, 1984). There is now general agreement that damage to the cell membrane constitutes alpha toxin's primary effect on cells. However there is still no unambiguous answer to the question of how alpha toxin acts at the cell membrane level. In the quest for an answer to this intriguing problem, much work has been done using the rabbit erythrocyte. The distinctive

susceptibility of these erythrocytes to the effects of alpha toxin and the ease and economy with which they can be obtained, has made this cell type a favourite model for studying the mode of action of this toxin.

Hemolytic events

Mangalo and Renaud (1959) using a turbidometric assay, measured alpha toxin mediated hemolysis of a suspension of erythrocytes. They found that the time required to lyse 50 percent of the suspension is directly related to the concentration of alpha toxin added. Lominski and Arbuthnott (1962) determined that the rate of hemolysis depends on the concentration of toxin added to the suspension. Studies on the rate of alpha toxin mediated lysis of rabbit erythrocytes revealed that the lytic phenomenon could be described by a sigmoidal curve (Cooper *et al.*, 1964a, b; Madoff *et al.*, 1964; Marucci, 1963a, b). Three stages in the time course of hemolysis could be identified; a pre- lytic lag phase, a lytic phase and a tailing off or slowing down of hemolysis. In the pre- lytic lag phase, alpha toxin is rapidly adsorbed onto the erythrocyte membrane. This bound toxin reaches a maximum during the greatest rate of lysis followed by a gradual decrease after lysis (Klainer *et al.*, 1964). After the binding of toxin to the membrane, the first detectable event is the release of potassium ions. Cassidy and Harshman (1976a, b) demonstrated the prelytic release of ^{86}Rb and have shown that this release closely parallels the binding of ^{125}I alpha toxin at 20 C. At 0 C the two events were found to be separate. Intracellular ^{86}Rb, an analog of potassium, was released approximately 40 minutes after the addition of alpha toxin. The lytic phase represented a time of rapid release of hemoglobin from the damaged erythrocytes (Cooper *et al.*, 1964a, b; Lominski and Arbuthnott, 1962).

Marucci (1963b) identified two steps in the hemolytic process. The first step involved the interaction of alpha toxin with the erythrocyte. The second step which was an intrinsic reaction of the damaged erythrocyte and occured without further participation of the toxin, led to the release of the hemoglobin. The first step could be arrested by the addition of antitoxin. At the second step, the erythrocytes were committed to lyse and the addition of antitoxin had no effect. Lo and Fackrell (1979) also identified these two steps, but, they found that both steps could be blocked by specific antibodies.

Mode of action

In attempts to determine the mode of action of alpha toxin, studies have been concentrated in the following three areas (1) the identification of enzymatic activities of the toxin, (2) morphological and biochemical studies for characterization of the structural membrane damage induced by the toxin, and, (3) studies concerning binding of the toxin to specific receptors on the cell surface.

The enzymatic mode of action for alpha toxin was first implicated in the early 1930's by Forssman (1933, 1934 a, b). However, in the late 1930's Levine (1938, 1939) demonstrated that the hemolytic, dermonecrotic, and lethal effects of alpha toxin could be adsorbed by concentrated suspensions of erythrocytes. This lead to the suggestion that alpha toxin and its substrate reacted stoichiometrically following the Freundlich adsorption isotherm and therefore, alpha toxin was not an enzyme. These contradictory findings were dismissed by Forssman (1939) as due to differences in technique. Robinson *et al.* (1960) by use of electrophoresis were unable to separate proteolytic and lytic activity of alpha toxin. As a result, they also suggested that alpha toxin is a protease although no strict kinetics were

carried out. In 1962, Lominski and Arbuthnott studied the kinetics of alpha toxin mediated hemolysis of rabbit erythrocytes and found two pieces of evidence that supported the enzymatic hypothesis. Firstly, they found that all the hemolytic activities were recovered in the supernatant after lysis. This suggests that the toxin acts as a catalyst. Secondly, the plot of the rate of hemolysis versus toxin concentration was similar to that of an enzyme reaction. Marucci (1963a, b) confirmed the first piece of evidence but argued that the phenomenon may simply reflect a situation of toxin in excess. Bernheimer (1970) compared various enzymatic and non- enzymatic lytic agents, by plotting the percent of lytic agent versus the log of erythrocyte concentration. It was found that alpha toxin conformed to the enzymatic curve. However, no conclusions could be drawn because beta toxin did not show a similar relationship although it is an enzyme. Wiseman and Caird (1970, 1972) and Wiseman *et al* (1975) presented supporting evidence for this proteolytic mechanism of action. They found that proteinaceous materials, monitored as soluble nitrogen, were released from erythrocytes and erythrocyte membranes after exposure to alpha toxin. Nitrogen release from erythrocyte membranes of different species was directly correlated with hemolytic sensitivity (Wiseman *et al.*, 1975). In addition it was demonstrated that alpha toxin can be activated by immobilized trypsin to hydrolyze tosyl arginine methyl ester (TAME) a property which the native toxin does not exhibit. The Km of the toxin was different from that of trypsin although the Vmax was the same for this substrate. This toxin was shown to hydrolyse 9 percent of the total protein content of the erythrocyte membrane. These researchers suggested that the native toxin (protoxin) is activated to become a protease by endogenous proteolytic activity in the membrane attacked (Wiseman *et al.*, 1975; Wiseman, 1975). The proteolytic activity of the cells of each species was directly correlated with the

sensitivity to alpha toxin (Morrison and Neurath, 1953; Moore *et al.*, 1970). Szmigielski and Harshman (1978) demonstrated that preincubation of alpha toxin with live suspended fibroblasts enhanced the hemolytic activity of the toxin, a finding which lends support to Wiseman's hypothesis.

In contrast to these studies just outlined, several investigators were unable to verify the proteolytic mode of action of alpha toxin. Freer *et al.*, (1973) were unable to demonstrate which membrane protein is the substrate of alpha toxin's proteolytic activity. PAGE gels of ghosts exposed to alpha toxin did not detect hydrolysis of any major membrane protein. In addition, these authors could not inhibit alpha toxin mediated hemolysis of erythrocytes by the protease inhibitor phenyl methane sulfonylfluoride, did not find a reduction of sedimentable protein and found that the freeze etch patterns of alpha toxin treated ghosts are not similar to those obtained using known proteases. Wiseman (1975) has suggested that the erythrocyte ghost preparations of Freer *et al.* (1973) did not contain the membrane protease responsible for the activation of alpha toxin. In addition, Madoff *et al.*, (1964) and Cassidy and Harshman (1976a, b) showed that the toxin is consumed in the lytic process.

In opposition to the enzymatic mode of action of alpha toxin is the view that emphasizes the surface activity of the toxin in biological membranes. A number of groups, (Cassidy *et al.*, 1974; Freer *et al.*, 1973; Weissmann *et al.*, 1966) demonstrated that alpha toxin disrupted liposomes composed of various mixtures of phospholipids and cholesterol. This disruption resulted in the release of marker molecules such as glucose, phosphate and chromate from the liposomes. Furthermore, this interaction with liposomes, as with interactions with erythrocyte ghosts, resulted in the loss of hemolytic activity. Liposomes prepared from human erythrocytes were found to be as sensitive to alpha toxin as those prepared from

rabbit erythrocytes. Since the rabbit erythrocyte is 100 times more sensitive to alpha toxin than the human erythrocyte, this indicates that a lipid component alone does not account for toxin mediated hemolysis. In these studies, high levels of toxin (15 to 30 ug per ml) were used. These levels are much higher than the amount needed to lyse erythrocytes (0.1 to 0.2 ug per ml) (Cassidy *et al.*, 1974).

This hydrophobic interaction between the toxin and membrane lipids in general was confirmed by studies on mixed lipid monolayers (Buckelew and Colacicco 1971). They found that in the absence of lipid, the toxin spreads as a film at an air water interface. When lipid was present, the toxin penetrated the lipid monolayers to differing degrees. Freer *et al.*, (1968) demonstrated that native 3S alpha toxin polymerizes to the 12S form on the surfaces intact cells and liposomes. These 12S forms of the toxin were morphologically indistinguishable from those that occur naturally in toxin preparations, or, those that resulted from heat treatment at 60 C. Bernheimer (1974) also observed this ring formation on rabbit, human, horse and guinea pig erythrocyte membranes, platelet membranes, rat hepatocyte plasma membranes, and lysosomal membranes prepared from rabbit polymorphonuclear leukocytes. However, these ring structures were not seen on the membranes of various bacterial species. Freeze etching studies revealed that the 12S ringlike structures penetrate the hydrophobic lipid regions of the platelet membrane (Bernheimer *et al.*, 1972) and that significant changes occur in the hydrophobic fracture plane of rabbit erythrocyte membranes that were exposed to the toxin (Freer *et al.*, 1973). By use of the detergent Triton X-100, Fussle *et al.*, (1981) isolated the ringlike structures from toxin treated rabbit and human membranes. These structures appeared in the electron microscope as hollow cylindrical structures with outer and inner diameters of 8-10 and 3-5 nanometers respectively. After boiling in SDS, SDS-PAGE performed on the 200 KD complexes revealed

that dissociation occurred yielding native toxin (34KD) monomers. This implied that the cylindrical structures are hexamers of the native toxin. Bhakdi *et al.*, (1981) and Fussle *et al.*, (1981) found that native toxin oligomerized into active hexamers in the presence of sodium deoxycholate micelles only. These hexamers when extracted, bound lipid and could be incorporated into artificial lecithin lipid vesicles.

Fussle *et al.*, (1981) using prelabelled and resealed ghosts treated with alpha toxin hexamers, revealed that the size of the lesions indicated by release of marker molecules coincided with the ultrastructural data presented in the same study and by Freer *et al.*, (1968). In the same study, Fussle *et al.*, (1981) suggested that human erythrocytes contain a protein that repairs the damage done by alpha toxin, and that this "repair protein" is absent in rabbit erythrocytes.

Harshman (1979) using low levels of alpha toxin (0.1 to 0.2 ug/ ml) did not observe ring formation although lysis was evident. Alpha toxin has been shown to enhance transbilayer reorientation of the phospholipid lysophosphatidylcholine as well as disrupt assymmetry of phosphatidyl- ethanolamine and phosphatidylcholine in erythrocyte membranes (Schneider *et al.*, 1986). Sheep erythrocytes, when treated with beta toxin, a phospholipase C, become resistant to hemolysis by alpha toxin (Elias and Kofer, 1980; Christie and Graydon, 1941). The reason for this antagonism is as yet unknown. The mode of action of alpha toxin may involve the lipid of the target cell membrane. Recent evidence has indicated that the toxin molecule undergoes a change when the hexamer is formed. A conformational change occurs concomitant with the transformation of the monomer to the hexamer (Ikigai and Nakae, 1985). The secondary and tertiary structure of the alpha toxin molecule has been described by Tobkes *et al.*, (1985). They demonstrated by circular dichroism spectra, that a high proportion of the molecule, in both

monomeric and hexameric forms, consists of beta structure (approximately 68 percent); beta sheet (approximately 55 percent) and beta turns (approximately 11 percent). There was little alpha helix structure noted (approximately 6 percent) and some random coil or unassigned structure (approximately 26 percent). The intramembranous section of the hexamer was suggested to be composed of domains of anti- parallel beta sheet that surround a central pore. Tobkes *et al.*, (1985) have presented a simple working model for the assembly of alpha toxin into lipid bilayers. They suggest that the tertiary structure of alpha toxin changes. The monomer contains two main domains connected by a hinge. The molecule turns partially inside out before it forms a hexamer. This change causes hydrophilic residues that were originally on the surface of the monomer, to line the pore or be involved in subunit- subunit and domain- domain interactions. The change in the molecule also reveals hydrophobic residues that had been masked between the two domains of the monomer. These residues interact with the hydrophobic core of the cell membrane. This model is supported in part by data presented by Gray and Kehoe (1984) who sequenced the alpha toxin gene. The sequence analysis indicates that in the region from residues 119 to 143 there is 32 percent glycine. This region may constitute the hinge suggested by Tobkes *et al.*, (1985).

It has become evident that the disruption of the target cell membrane is not explained solely by the surface- active properties of alpha toxin. The wide range of sensitivities of cells to damage by alpha toxin is not explained by this model (Arbuthnott *et al.*, 1973).

Cassidy and Harshman (1976a, b) and Barei and Fackrell (1979) also observed ringlike structures on membranes. However, they argued that these structures, seen only at high concentrations of alpha toxin, were unrelated to the binding of

alpha toxin to high affinity receptors at low, but lytic concentrations of the toxin. In addition, it must also be noted that the EM studies done by Freer *et al.*, (1973) in which hexamers were observed in toxin treated membranes involved the use of erythrocyte ghosts rather than intact erythrocytes. It has been shown by both Arbuthnott *et al.*, (1974) and Cassidy *et al.*, (1974) that only about 5 percent of added toxin binds to intact erythrocytes, whereas 60–70 percent added alpha toxin binds to osmotically prepared ghosts. It is possible that alpha toxin binds non–specifically to ghosts, leading to ring formation. In addition, the surface activity mode of action theory cannot explain the variability among erythrocytes of different species which are susceptible in varying degrees to alpha toxin (Bernheimer, 1965; Cooper *et al.*, 1966). Since Cassidy *et al.*, (1974) did not find the species specificity for lipids that exists for erythrocytes, this phenomenon may instead be related to the interaction with specific receptors on the erythrocyte membrane.

For a number of years various researchers have postulated the existence of specific receptors for alpha toxin on the erythrocyte membrane (Austin and Fackrell, 1984; Barei and Fackrell, 1979; Cassidy and Harshman, 1973; 1976a, b, 1979; Cooper *et al.*, 1964a, b; Kato *et al.*, 1975; Kato and Naiki, 1976; Klainer *et al.*, 1964, 1972; Lo and Fackrell, 1979; Maharaj and Fackrell, 1980; Marucci, 1963a; Wiseman and Caird, 1972; Wiseman *et al.*, 1975).

Cassidy and Harshman (1976a, b) found that ^{125}I monoiodinated alpha toxin bound irreversibly to rabbit red blood cells. The extent of the binding was greater than that seen for human red blood cells, and was correlated to the extent of subsequent lysis of these cells. They also found that binding occured in a time and temperature dependent fashion. When alpha toxin was added to rabbit erythrocytes at 20 C, binding and lysis occured almost simultaneously. However,

when the experiment was repeated at 0 C, binding preceeded lysis by almost 40 minutes. These authors, using the technique of Scatchard analysis, determined that there were approximately 5000 high affinity binding sites per rabbit erythrocyte. These high affinity binding sites, or receptors, were suspected to be proteinaceous in nature since pronase treatment of the erythrocytes resulted in a decreased number of binding sites. This finding is in agreement with those of Kato *et al.*, (1975) who found that pretreatment of rabbit erythrocytes with pronase, decreased their sensitivity to alpha toxin by 97 percent. Cassidy and Harshman (1976a, b) were also unable to detect any such receptors on the human erythrocyte. Barei and Fackrell (1979) also by Scatchard analysis, estimated the number of high affinity binding sites on the rabbit red blood cell to be 125,000. The discrepancy in the numbers generated by the two groups may be due to the fact that Harshman's study was done with ^{125}–I alpha toxin which retained 10 percent of its hemolytic activity, while Fackrell's group used fluorescein labelled toxoid. This residual activity of Harshman's pereparation may have caused release of the bound toxin, which is the case with the active toxin (Klainer *et al.*, 1964). This release of bound toxin would have effectively prevented the establishment of true equilibrium. Barei and Fackrell (1979) also established a correlation between the number of receptors on erythrocytes from different species and their hemolytic sensitivity to alpha toxin. In addition they found that hemolytic sensitivity was independent of the number of receptors if this value is less than 37,000 per red blood cell. In 1979, Cassidy and Harshman presented additional evidence for the specific interaction of ^{125}–I alpha toxin with an erythrocyte membrane protein. They isolated high molecular weight complexes (10^6) that were extremely stable to denaturation in SDS or urea solutions and proteolytic to degradation. However, treatment of the complex with SDS at 100 C resulted in the label co–migrating with

native toxin. This group presented a model that not only explained the formation of hexamers from initially established monomer complexes, but also accounted for the 12S forms and the apparent loss of receptors that occur when rabbit erythrocytes are preincubated with sub– saturating concentrations of alpha toxin. It was claimed that the high affinity receptors function merely to increase local concentrations of alpha toxin on the rabbit erythrocyte cell surface. This explains the low concentrations of alpha toxin needed to lyse rabbit erythrocytes as compared to those of other species. Kato and Naiki (1976) suggested that the receptor is an N–acetyl–glucosaminyl– ganglioside residue since nanogram amounts of this compound inhibited lysis of rabbit erythrocytes by alpha toxin. This finding could not be substantiated by other groups (Fackrell, unpublished data; Bernheimer, personal communication). Other attempts to identify a specific receptor for alpha toxin were also done by inhibition studies. Kato *et al.*, (1975) and Raff *et al.*, (1977) found that flavin mononucleotides and related flavines inhibited alpha toxin mediated lysis of rabbit red blood cells. This finding was confirmed in this laboratory (Fackrell, unpublished data). Bernheimer and Avigad (1980) also reported that the transmembrane protein glycophorin, isolated from human erythrocytes, inhibited alpha toxin– mediated lysis of rabbit red blood cells. The inhibitory effect of these substances, however, might merely be due to non–specific neutralization of the toxin's surface active properties by ionic or hydrophobic interactions (Bernheimer, personal communication; Thelestam, 1983a).

Maharaj and Fackrell (1980) presented several pieces of evidence which indicate that the alpha toxin receptor is the integral erythrocyte membrane glycoprotein referred to as band 3 (Steck *et al.*, 1971). Firstly, it was found that band 3 is sensitive to pronase and alpha chymotrypsin digestion. This corroborates the findings of Cassidy and Harshman (1976a, b) and Kato *et al.*, (1975) who found

that the alpha toxin receptor is pronase sensitive. Secondly, erythrocytes preincubated with the lectin con A, are protected from lysis by alpha toxin. Since con A binds to band 3, this implicates band 3 as the receptor for alpha toxin. In addition, the sugars glucose and mannose, which selectively inhibit con A reduce this protective effect. Thirdly, purified band three reduced the hemolytic activity of alpha toxin, and, antibodies to band 3 protect rabbit erythrocytes from lysis. Recent work in our laboratory (Simpson, 1986, MSc thesis) has further narrowed down the location of the receptor in the rabbit erythrocyte band 3. He demonstrated that a receptor for alpha toxin is in the 35 kD C- terminal chymotryptic fragment of band three. Extensive proteolytic digestion and hydrazine destruction of the polypeptide of this fragment had no effect on the receptor. However, neuraminidase digestion and sialic acid residue modification affected the binding of alpha toxin to the receptor. This suggests that the carbohydrate moiety of rabbit erythrocyte band 3 is a receptor for alpha toxin. Now, since band 3 occurs in human as well as rabbit erythrocytes, the findings of Maharaj and Fackrell (1980) can only be explained if the band 3 receptor on the human erythrocyte is somehow masked, or altered. This is in agreement with the fact trypsin and pronase treated human red blood cells have an increased sensitivity to alpha toxin. Treatment with these enzymes may have resulted in the unmasking of the carbohydrate moiety of human erythrocyte band 3 (Simpson, 1986, MSc thesis).

In 1979, Lo and Fackrell, using antigenic determinants as reference points, presented immunologic evidence that alpha toxin is oriented in a specific manner on rabbit red blood cell membranes, and thus, posseses a membrane binding region. This is in turn is consistent with the idea of a specific receptor for alpha toxin. In addition, in this study, it was demonstrated that anti- alpha toxin antibodies can

be separated into two distinct populations. One population, the anti-binding antibodies (AB-Ab) prevents the binding of alpha toxin to the membrane. The second population causes neutralization of the toxin after it is bound on the membrane. Since this phenomenon could be measured by indirect hemagglutination, this population of antibodies is referred to as Indirect Hemagglutinating antibodies (IHA-Ab). These results suggest that binding of the toxin and subsequent lysis are separate events, and thus reiterates the findings of Cassidy and Harshman (1976a, b) and Madoff *et al.*, (1964).

Further examination of the two anti-toxin populations (Lo, 1984, PhD dissertation) indicate that neutralization of alpha toxin depends on the concentration of antibodies, with a linear dependence for IHA-Ab and, a complex non-additive relationship for AB-Ab. This infers that only one IHA determinant needs to be blocked to effect neutralization. In addition, it seems as if the protective effect of IHA antibodies is reduced if added after the lag phase, a finding which further implies that at least two separate lytic events occur after binding of the toxin.

Before this section is concluded, it should be noted that although three groups have now independently demonstrated the existence of receptors for alpha toxin on the red blood cell membrane, this concept is not universally accepted. Freer *et al.*; (as reported by Thelestam, 1983) found that binding of ^{125}-I alpha toxin to rabbit erythrocytes is temperature dependent between 0 and 45 C with a sharp transition at 12 to 15 C which allows adsorbed toxin to effect lysis. They have also been unable to demonstrate any saturation of binding sites, or, the existence of high affinity binding sites. These findings support the theory of adsorption of alpha toxin to membranes rather than binding of the toxin to specific receptors. Although these findings do not completely rule out the possibility of receptors,

Freer and his collegues believe instead, that the sensitivity of rabbit erythrocytes is due to peculiarities connected with lipid transitions in the membrane. They have found that alpha toxin can penetrate mixed lipid films of 17 dynes per cm, but not 34 dynes per cm (Freer *et al.*, 1968). Since the surface pressure in the lipid bilayer of intact human erythrocyte membranes is in the range of 31 to 34 dynes per cm, this would explain the low sensitivity of human erythrocytes to alpha toxin. However, since erythrocytes of other species, particularly those of the rabbit have not been evaluated in terms of their surface pressure, this concept remains speculative at this point.

From the above findings, it can be seen that although much progress has been made in understanding the biological and immunochemical properties and behaviour of alpha toxin, there are still many questions that remain to be answered.

MATERIALS AND METHODS

Biological Methods

Cultures

Cultures of *Staphylococcus aureus* strain *Wood 46* were originally obtained from Gow and Robinson (1969) at the University of Western Ontario. This strain fulfilled the criteria as set by Baird– Parker (1972; 1974) and Kloos and Schleifer (1986) to be classified as *Staphylococcus aureus*. Phage– typing of this strain was performed by the Canadian Communicable Disease Centre in Ottawa, Canada. Stock cultures were maintained by lyophilization, freezing at –90° C, or in liquid nitrogen. When neccessary, the cultures were grown on rabbit blood agar plates that were incubated at 37° C for 36 hours in an atmosphere of 10 percent CO_2 and 90 percent air. The colonies that demonstrated the largest zones of hemolysis were subcultured on rabbit blood agar plates and subsequently used to produce alpha toxin.

Production and concentration of crude alpha toxin

Crude alpha toxin was produced according to the method of Wiseman *et al.*, (1975). A 10 mL aliquot of an 18 hour culture of *Staphylococcus aureus* strain *Wood 46* was used to inoculate each of 24 one litre flasks each containing 500 mL of sterile Dolman Wilson (Dolman and Wilson, 1940) medium. The flasks were incubated at 37° C in an atmosphere of 10 percent CO_2 and 90 percent air, with constant shaking at approximately 150 rotations per minute. After 36 hours, the culture was centrifuged at 10,000g at 0° C for 20 minutes. The supernatant which contain the alpha toxin, was immediately frozen at –20° C.

Two methods were used to concentrate the crude alpha toxin. The first method was that of Wiseman *et al.*, (1975). The previously frozen culture supernatant was thawed and the pH adjusted to 4.0. The alpha toxin protein was then precipitated overnight by addition of cold methanol (−20° C) to a final concentration of 35 percent (V/V). The precipitate was then collected by centrifugation (10,000g at 0° C for 20 minutes) and redissolved in approximately 500 mL phosphate buffered saline. To this dissolved precipitate, a saturated ammonium sulphate solution was added to a final concentration of 40 percent (V/V). The mixture was then left at room temperature for 8 to 12 hours and then centrifuged as before and the supernatant retained. More saturated ammonium sulphate solution was then added to the supernatant to a final concentration of 60 percent (V/V). This mixture was allowed to sit at room temperature for approximately 12 hours, then centrifuged and the pellet saved. This precipitate which contained semi– purified alpha toxin in a highly concentrated form, was dissolved in phosphate buffered saline and dialyzed versus the same buffer overnight at 4° C. This yielded biologically active toxin. Toxin stored under 60 percent ammonium sulphate retained its biological activity indefinitely.

The second method used to concentrate crude alpha toxin was that of Dalen (1976c). The previously frozen supernatant was thawed, adjusted to pH 4.0 to 5.0 and incubated at 60° C for 30 minutes. The precipitate was collected by centrifugation (10,000g at 0° C for 20 minutes) washed twice in 0.03 M borate buffer pH 8.6, and finally dissolved in 0.03 M borate buffer, pH 8.6 containing 8 Molar urea.

Purification of alpha toxin

Two different methods were used to purify alpha toxin. The first method involved the principle of affinity chromatography. Polyclonal antibodies raised against purified alpha toxin were linked (with cyanogen bromide) to either Sepharose 4B (Pharmacia) beads or, linked (with N–ethyl–N'–(3–dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Sigma Chemical Company)) to CM Bio–Gel A (Bio–Rad Laboratories) beads. Concentrated semi–purified alpha toxin dissolved in PBS was then added to the prepared toxin– bead complex. The mixture was then washed three times in 4 volumes of PBS to remove unbound material. The alpha toxin was then eluted by the addition of glycine– HCl and dialyzed versus PBS. Alpha toxin prepared by this method was not biologically active.

Alpha toxin was also purified according to the method of Wiseman *et al.*, (1975). The concentrated semi– purified alpha toxin was extensively dialyzed versus Hallander's buffer at 4° C. The toxin was then applied to a Sephadex G–75 column and eluted with the same buffer. The fractions that were collected, were assayed for alpha toxin content by addition of 100 ul aliquots to 50 ul of a 2 – percent suspension of rabbit erythrocytes in PBS. Fractions that showed complete hemolysis were pooled. Alpha toxin prepared in this manner was greatly diluted. The toxin was concentrated by use of an Amicon Ultrafiltration system equipped with a PM 10 Diaflo ultrafiltration membrane (Amicon Corporation USA).

Preparation of alpha toxoid

Alpha toxin, either in its crude, semi- purified, or purified form was rendered non- hemolytic by immersion in a water bath at 60° C for 30 minutes. The resultant turbid material was clarified by centrifugation at 8000g for 1 hour. The usage of the term "toxoid" is in accordance with that of Burnet (1931) who restricted this term to represent physically denatured, non- toxic forms and applied the term "anatoxin" to represent formalin detoxified forms.

Erythrocytes

New Zealand White rabbits were maintained and cared for in accordance with the publication, "The principle of care of experimental animals - A guide for Canada". These animals were bled by cardiac puncture, or, from the ear vein and the blood collected into 125 mL Erylenmeyer flasks containing approximately 10 ml of glass or plastic beads. The flasks were shaken to prevent clotting. The erythrocytes were packed by centrifugation at 600g for 6 minutes and the serum and buffy coat removed by aspiration. The erythrocytes were strained through a double layer of cheese cloth and then washed four times in six volumes of PBS (600g for 6 minutes). The washed erythrocytes were suspended to a 2 percent hematocrit in PBS for use in the kinetic hemolysis assay. These erythrocytes were stored at 4° C, and, were discarded every day. Erythrocytes that were kept for longer periods of time were stored in an equal volume of Alsever's solution (Carpenter, 1975).

Erythrocyte membranes were prepared according to the method of Dodge *et al.*, (1963).

The Hemolytic Titration Assay

The 50 percent endpoint method (Wiseman and Caird, 1972) was modified (Lo and Fackrell, 1979) for use with Microtitre plates (Cooke Laboratory Products) by proportional reduction of the reagent volumes. Fifty microlitres of alpha toxin was serially diluted in 50 ul of PBS in a Microtitre plate with U– shaped wells. Fifty microlitres of a 2 percent suspension of rabbit erythrocytes (in PBS) was then added to each well, and the mixture was incubated at 37° C for 1 hour. Fifty microlitres of PBS was then added to each well, and the plate was shaken gently. After centrifugation at 600g for 5 minutes, 100 ul of the supernatant was diluted 1:3 with PBS, and the absorbance was measured at 541 nm (1 centimetre light path). The hemoglobin content of the supernatants was translated into percent hemolysis by comparison to standards. The standards were prepared from a lysate of 2 percent rabbit erythrocytes in milliosmolar buffer. This comparison and the determination of the 50 percent endpoint were done using a computer program designed for linear regression calculations. One hemolytic unit (HU) is defined as the concentration of alpha toxin that produces 50 percent hemolysis, under these specified conditions.

The Indirect Hemagglutination (IHA) Test

The IHA test was performed according to the method of Lo and Fackrell (1979). Fifty microlitres of serum was serially diluted in 50 microlitres PBS in a Microtitre plate with U– shaped wells. Fifty microlitres of a 1 percent suspension of toxoid– coated erythrocytes was then added to each well. The plates were then incubated at 4° C for 90 minutes. Formation of a large ring of agglutinated cells indicated a positive IHA reaction. Formation of a small ring or a button of cells

indicated a negative IHA test. The IHA titre of any given serum preparation is defined as the reciprocal value of the lowest serum concentration giving a positive reaction. In each IHA test, the following controls were included: The first control was for the toxoid– coated erythrocytes. Fifty microlitres of these erythrocytes were mixed with 50 ul PBS. Tests were rejected if spontaneous hemagglutination occured. The second control was for the sera being tested. Fifty microlitres of each test serum was mixed with 50 ul of a 1 percent suspension of uncoated rabbit erythrocytes. Any sign of hemagglutination, which indicated the presence of antibodies that cross react with the erythrocytes, invalidated the IHA test. These cross– reacting antibodies were removed by adsorption with 2 volumes of packed erythrocytes at 4 C for 5 minutes. The sera were repeatedly adsorbed until no agglutination occured when titrated with uncoated erythrocytes. The IHA test was then repeated with the adsorbed sera. In addition to these controls, known positive and negative sera were included in each test to ensure reproducibility.

Production of antisera

New Zealand white rabbits were hyperimmunized with purified alpha toxoid at a concentration of 2 mg/ml in PBS mixed with complete Freund's adjuvant at a ratio of 1:1 (V/V). One millilitre of the antigen– adjuvant mixture was administered intra– muscularly into each hind leg. Booster doses were given once a month and the rabbits were bled every week for several weeks.

Purification of Immunoglobulin G

The sera of some animals were purified to immunoglobulin G (IgG) fractions

according to the method of Campbell *et al.*, (1970). This method involved the initial precipitation of gamma globulins from the sera by the addition of saturated ammonium sulphate to a final concentration of 33.3 percent (V/V). The ammonium sulphate solution was adjusted to pH 8.0 with 2 N NaOH and then added dropwise to the sera which was constantly stirred by a magnetic stirrer. The mixture was allowed to sit overnight at room temperature and then was centrifuged (2000g for 15 minutes) and the supernatant discarded. The precipitate was then dissolved in borate buffered saline pH 8.5 and the precipitation procedure repeated for a total of three times. The final precipitate was then dissolved in a small amount of borate buffered saline and the mixture was extensively dialyzed versus the same buffer. The solution was then stored at 4° C. Furthef purification was accomplished by ion exchange chromatography using DEAE cellulose. The column was equilibrated with 0.01 M phosphate buffer pH 7.5, and the IgG eluted with the same buffer. Fractions showing the highest absorbance at 280 nm were pooled and concentrated with an Amicon ultrafiltration system equipped with a Diaflo PM 30 membrane. The identity of the anti- alpha toxin IgG fraction was confirmed by immunodiffusion versus alpha toxin and goat anti-rabbit IgG.

Preparation of toxoid- coated erythrocytes (immunoadsorbent)

Rabbit erythrocytes were coated with alpha toxoid according to the method of Lo and Fackrell (1979). Rabbit erythrocytes that were previously washed with PBS were adjusted to a final concentration of 10 percent (V/V) in an alpha toxoid solution (1 mg/ mL in PBS). The mixture was periodically shaken over a 30 minute incubation period at room temperature. Unbound materials were then

removed by washing the coated erythrocytes (600g for 10 minutes) three times in PBS. The bound toxoid was then covalently linked to the carrier erythrocytes via the bifunctional reagent glutaraldehyde. The coated erythrocytes were adjusted to a 2 percent (V/V) suspension in fresh 0.1 percent (W/V) glutaraldehyde solution prepared in PBS. The mixture was resuspended for 30 minutes at room temperature and then washed as before in PBS (3x). To block excess aldehyde groups, the coated erythrocytes were resuspended to a final concentration of 20 percent (V/V) in 0.1 M monoethanolamine solution prepared in PBS, for 1 hour at room temperature. Finally, the coated erythrocytes were washed three times in PBS and stored at 4° C with 0.01 percent (W/V) sodium azide as preservative.

Fractionation of anti– alpha toxin antibodies

The anti– alpha toxin antibodies were separated into IHA and AB populations according to the method of Lo and Fackrell (1979). Rabbit anti– alpha toxin sera was mixed with an excess of the immunoadsorbent for 1 hour at room temperature or, overnight at 4° C. The mixture was centrifuged at 800g for 10 minutes. The supernatant was then checked in the IHA test, and if still positive, it was readsorbed until it became negative. This supernatant which contained antibinding antibodies (AB–Ab) was then clarified by centrifugation at 40,000g for 1 hour.

The indirect hemagglutinating antibody (IHA)– bearing immunoadsorbent was washed twice in PBS to remove unbound serum components and then resuspended in a small amount of PBS. This mixture was heated at 56 C for 20 minutes with constant agitation, and then centrifuged at 56 C (800g for 10 minutes) in covered centrifuge tubes. The supernatant which contained the eluted IHA antibodies was clarified as above.

The Kinetic Hemolysis Assay

This assay was performed in a Perkin– Elmer Coleman 124 spectrophotometer, and the data recorded, via an analog to digital converter (Glasgow and Fackrell, 1984) by an Apple II+ computer. A BASIC program called "Kinetics" (Fackrell and Burgess, unpublished data) analyzed the data and calculated several parameters. These include the length of the pre–lytic lag phase, the time to 50 percent hemolysis, the time to completion, the average and maximum rates of lysis and the percentage of unlysed cells at completion. In this assay, 200 ul of a 2 percent suspension of rabbit erythrocytes was added to 3 mL of PBS in a cuvette with a 1 cm light path. The required volume of alpha toxin (the volume of toxin may vary from 5 to 50 ul depending on the protein concentration) was then added and the suspension thoroughly mixed with a pipette. The alpha toxin– mediated lysis of the erythrocytes in suspension was measured by the decrease in absorbance at 650 nm. In order to determine any inhibition of the toxin's activity, the sample (antibody or CNBr digested toxin) was preincubated with the toxin and incubated in an ice bath for approximately 10 minutes just prior to use.

ELISA for detection of alpha toxin

This assay was performed according to the method of Surujballi and Fackrell (1984).

Reagents and equipment: The buffers used in this assay were as follows: coating buffer, 0.05 M sodium carbonate, pH 9.6; substrate buffer, 0.2 M Tris adjusted to pH 9.8 with 0.2 M HCl; phosphate buffered saline (PBS) (0.01 M sodium phosphate

buffer supplemented with 0.15 M sodium chloride, pH 7.4) and PBS containing 0.05 percent Tween- 20 (PBS- Tween). Sodium azide (0.2 percent (W/ V) and pentachlorophenol (0.02 percent) were added as preservatives in all buffers.

The enzyme used in this ELISA was alkaline phosphatase (EC 3.1.3.1, type VII, Sigma Chemical Company). The substrate *p*- nitrophenyl phosphate (Sigma Chemical Company) was used at a concentration of 1.0 mg per ml. Hydrolyzed substrate was measured at 405 nm with a Perkin- Elmer Coleman 124 spectrophotometer. Protein A was obtained from Sigma Chemical Company.

In this assay, two adsorbent surfaces were used. The antibody was adsorbed onto either polystyrene microtitre plates (Cooke Microtitre plates obtained from Fisher Scientific Limited) or polystyrene (5 percent (W/V) in methylene chloride)- coated steel beads (4 mm diameter, Kidde Recreation Products Ltd., Cambridge, Ontario) (Smith and Gehle, 1980).

Conjugation of anti- alpha toxin with alkaline phosphatase: In accordance with Clark and Adams (1977) all references to the use of this antibody- enzyme conjugate are in terms of dilutions rather than absolute concentrations. This perspective accomodated volume changes and possible immunoglobulin losses during the conjugation procedure. The enzyme, alkaline phosphatase, was conjugated with purified anti- alpha toxin immunoglobulin G, by a modification of the method of Clark and Adams (1977). Two milligrams of alkaline phosphatase (EC 3.1.3.1, type VII, Sigma Chemical Company) were dissolved in 1 mL of IgG solution (1 mg per ml, in coating buffer) and the mixture was dialyzed extensively against PBS (1 litre, four times) at 4° C. Fresh glutaraldehyde was then added to a 0.05 percent (W/V) final concentration, and the mixture was incubated at room temperature for 4 hours. Ten microlitres of monoethanolamine were then added, and the mixture was dialyzed against PBS (1 litre, four times) to which monoethanolamine was added (1

percent (V/V)).

Washing the adsorbent surfaces: The surfaces of the adsorbents were washed before use with 70 percent ethanol (two times) followed by PBS– Tween (three times). The Microtitre plates were washed by filling the wells with the appropriate reagent for three minutes with periodic agitation. The plate was then shaken dry and the procedure repeated. Washes were also done between reaction stages with PBS– Tween. These washes were performed with due care so as to avoid cross contamination of the wells. The polystyrene– coated steel beads were washed by the following procedure: a bead– transfer device (Smith and Gehle, 1980) was placed over the Microtitre plate containing the beads. When a ceramic magnet was placed on the transfer device, the magnetic attraction facilitated transfer of the beads to a clean Microtitre plate containing the wash reagent. The beads were retained in the wash reagent for 3 minutes with periodic agitation, and the entire procedure was repeated.

Detection of alpha toxin: The Microtitre plate was washed as described before. The wells were then filled with 200 ul of purified anti– alpha toxin IgG (in coating buffer) and the plate was covered and incubated for 2 to 6 hours at 37° C, or, overnight at 4° C. The wells were then decanted, washed four times, and filled with 200 ul of the test sample. The plate was covered, incubated overnight, decanted, and washed four times. The wells were next filled with 200 ul of the antibody– enzyme conjugate, and the plate was covered, incubated 4 to 8 hours at 37° C, and then decanted and washed four times. Finally, the wells were filled with 200 ul of the substrate solution and incubated at 37° C for 1 hour. The enzyme reaction was stopped by the addition of 50 ul of 3M NaOH to each well. The amount of hydrolyzed substrate in each well was then measured by reading the absorbance at 405 nm in the spectphotometer.

When the polystyrene– coated steel beads were used, the procedure of Smith and Gehle (1980) was followed except that the beads were pre– incubated with the anti– alpha toxin IgG solution. The incubation times and temperatures for the antigen, the antibody– enzyme conjugate, and the substrate were identical to those described for the Microtiter plate technique.

Controls: In this assay four controls were used, one to examine each of the four stages: (1) antibody coating, and addition of (2) antigen, (3) antibody– enzyme conjugate, and (4) substrate. In each control, PBS– Tween was substituted for the appropriate reagent, with the three other reagents added in their regular sequence. The highest control reading was always observed when the antigen was excluded, indicating non– specific adherence of the antibody– enzyme conjugate to the plastic. This highest control value was subtracted from all readings to give the absorbance values expressed in the results.

ELISA for the detection of anti– alpha toxin antibodies

Reagents and equipment: The buffers used in this assay were as follows: coating buffer, 0.05 M sodium carbonate at pH 9.6; substrate buffer for alkaline phosphatase, 0.2 M Tris adjusted to pH 9.8 with 0.2 M HCl; substrate buffer for horseradish peroxidase, 1 M citrate supplemented just prior to use with 0.04 percent hydrogen peroxide; phosphate buffered saline (PBS, 0.01 M sodium phosphate buffer, pH 7.4, supplemented with 0.15 M sodium chloride); PBS containing 0.05 percent Tween– 20 (PBS– Tween) and antibody buffer, 1 percent bovine serum albumin dissolved in PBS– Tween. PBS and PBS– Tween can be replaced with Tris buffered saline (TBS, 0.01 M Tris adjusted to pH 7.4 with HCl and supplemented with 0.15 M sodium chloride) and TBS containing 0.05 percent

Tween- 20 (TBS- Tween) respectively, without affecting the assay. Thimerosal (Sigma Chemical Company) (0.02 percent (W/ V)) was added as a preservative to all buffers.

This ELISA was performed in Microtitre plates. These plates were obtained from either Fisher Scientific Ltd. (Cooke microtitre plates, U bottomed) or from Gibco/BRL (Nunc microtitre plates, flat bottomed). The choice of plates has no effect on the sensitivity or variability of the assay. The enzyme- labelled antibody was either a commercial preparation of goat- anti rabbit IgG conjugated with horseradish peroxidase (Bio- Rad Laboratories) or, goat- anti mouse IgG conjugated with alkaline phosphatase (Bio- Rad Laboratories). The horseradish peroxidase substrate was 0.12 percent o-phenylenediamine (OPD) which in the presence of hydrogen peroxide (0.04 percent) is catalyzed oxidatively from the nearly colourless OPD solution to a yellowish- orange solution with an absorbance maximum of 490 nm. The alkaline phosphatase substrate, p- nitropheny! phosphate (PNPP) was used at a concentration of 1.0 mg per ml. PNPP is catalyzed to yield a yellow solution, p- nitrophenol (PNP) which has an absorbance maximum of 405 nm. The hydrolyzed substrates were measured at their respective wavelengths with a Microplate Autoreader (Bio- Tek Instruments).

Detection of anti- alpha toxin antibodies: The Microtitre plate was washed prior to use as outlined before. Alpha toxin dissolved in coating buffer was then added, 200 ul per well, and the plate was incubated for 2 to 6 hours at 37° C or, overnight at 4° C. The wells were decanted, washed four times, and then filled with 350 ul of blocking solution (1 percent BSA in PBS). The wells were then decanted, washed four times and then filled with 100 ul of either rabbit serum, mouse hybridoma tissue culture supernatant, or, mouse ascites fluid diluted in antibody buffer. The covered plate was then incubated overnight at room temperature,

decanted, and washed four times. The wells were then filled with 100 ul of the antibody– enzyme conjugate (diluted according to the manufacturer's instructions) the plate was covered, incubated 1 to 4 hours at RT, and then decanted and washed four times. The wells were filled 100 ul of the appropriate substrate solution and incubated at room temperature. The reaction was stopped after 30 minutes by the addition of 50 ul of 1 M sulphuric acid to each well that contain o–phenylenediamine ,or, 50 ul of 3 M sodium hydroxide to each well that contain alkaline phosphatase. The absorbance of each well was then measured with a Microplate Autoreader (Bio–Tek Instruments) at the appropriate wavelengths.

Controls: Five controls were used in this assay. Four of these controls examined each of the four stages of the assay: (1) antigen coating and addition of (2) antibody, (3) antibody– enzyme conjugate and (4) substrate. In each of these controls, PBS– Tween was substituted for the appropriate reagent, with the others added in their regular sequence. The additional control consisted of pre– immune rabbit serum or, ascites fluid obtained from pristane– primed BALB/ cbyj mice that were injected with myeloma cells. The highest control reading was always observed when the test sample was omitted, indicating non– specific adherence of the antibody– enzyme conjugate to the plastic. The highest control value was subtracted from all readings to give the absorbance values expressed in the results.

Competitive ELISA for the detection of immunologically reactive fragments of alpha toxin

This assay is performed in Microtitre plates as described for the ELISA for detection of intact alpha toxin. The plates were washed prior to use as described before. The wells were then coated with a solution of anti– alpha toxin antibodies

(1 mg per mL in coating buffer, 200 ul per well) and the plate incubated 4 to 6 hours at 37° C, or, overnight at 4° C. The wells were then decanted and washed four times. Samples of alpha toxin that were taken at intervals over the duration of the digestion course were then added to the wells in duplicate (200 ul per well) and the plate incubated at room temperature overnight. The wells were decanted and washed four times and then alpha toxin was added (1 mg per mL in PBS, 200 ul per well). The plate was then incubated at room temperature for two hours after which the wells were decanted and washed four times. The anti– alpha toxin – alkaline phosphatase conjugate was then added (200 ul per well) and the plate incubated at 37° C for 4 to 8 hours. The wells were then decanted, washed four times and the substrate solution (PNPP) was added, 200 ul per well. The plate was then incubated at 37° C for 1 hour after which the reaction was stopped by the addition of 50 ul of 3M sodium hydroxide to each well. The absorbance of each individual well was then measured at 405 nm.

SDS– PAGE/ enzyme– linked immunoelectrotransfer blot system

This procedure was performed according to the method of Towbin *et al.*, (1979) with some modifications.

Reagents and equipment: The buffers used in this procedure were as follows: transfer buffer, 0.025 M Tris/ 0.192 M glycine/ 20 percent (vol/vol) methanol at pH 8.3; the wash buffers were Tris buffered saline (TBS) 0.01 M Tris adjusted to pH 7.4 with HCl and supplemented with 0.15 M sodium chloride, and, TBS containing 0.05 percent Tween– 20 (TBS– Tween). The staining solution for the nitrocellulose membrane consisted of 0.1 percent amido black in 45 percent methanol/ 10 percent acetic acid. The destaining solution consisted of 90 percent methanol/ 2 percent

acetic acid. The anti– species (indicator) antibody– horseradish peroxidase conjugates were purchased from Bio Rad Laboratories. The substrate, 4–chloro–1–napthol, (Bio Rad Laboratories) was dissolved in 10 mL methanol (3 mg/ ml) just before use. This mixture was then dissolved in 50 mL TBS and supplemented with 0.04 percent hydrogen peroxide. The transfer chamber was similar to the one described by Towbin *et al.*, (1979) with the electrodes 7 cm apart.

Samples were separated with SDS– PAGE as described, except that 10 minutes before the end of the run the power was briefly disconnected and 10 ul of methyl green dye solution (equal parts of 0.1 percent aqueous methyl green and glycerol) were added to each well. The power was then reconnected and the run resumed until the methyl green entered the separating gel to a distance of approximately 3 to 4 millimeters. The methyl green transfers to the nitrocellulose membrane and serves as lane markers. After the electrophoresis was completed, the gel was equilibrated in approximately 200 mL of transfer buffer at room temperature for 20 minutes. During this time the nitrocellulose membrane (0.45 um pore size) (Bio–Rad Laboratories) and four pieces of Whatman # 1 filter paper which were cut to the same dimensions as the nitrocellulose membrane were also equilibrated in transfer buffer, with care being taken to exclude air bubbles. After equilibration, the the gel and the nitrocellulose membrane were sandwiched using the following protocol. The sheet of nitrocellulose was laid on a double layer of filter paper which was supported by a similar sized piece of Scotch Brite scouring pad. The gel to be transferred was put on the nitrocellulose membrane with care being taken to exclude all air bubbles. (This technique can be sucessfully accomplished by starting from the middle and then carefully lowering both ends of the gel simultaneously. Any trapped air bubbles can then be gently pushed to the edges

with a gloved finger). A double layer of filter paper was then placed on the gel. Finally another piece of Scotch Brite scouring pad was placed on the filter paper. This entire assembly was then sandwiched in a plastic casette which was then fastened to eliminate any possibility of slippage during the transfer. The casette was then placed in the transfer chamber filled with transfer buffer, with the nitrocellulose membrane facing the anode. The transfer chamber was then placed in an ice bath and a constant current of approximately 225 milliamperes applied for approximately 18 hours. The sandwich was then taken apart and the nitrocellulose membrane sliced as needed using the methyl green dye as lane markers. To estimate the efficiency of protein transfer, an appropriate strip of nitrocellulose membrane was then stained for 10 minutes in staining solution, and destained for 10 minutes in destaining solution. The protein bands appeared blue in a white background. The gel was also stained in Coomassie Blue and compared with the nitrocellulose strip to determine which bands were transferred. The nitrocellulose membrane strips that were needed for further participation in the ELISA were immersed in a 5 percent BSA (in TBS) solution for 1 hour at 37° C with gentle shaking. This procedure effectively blocked the remaining protein binding sites on the nitrocellulose membrane. The blocked nitrocellulose membrane strips were then incubated in the first antibody solution (1 to 20 dilution in TBS) overnight at room temperature with gentle shaking. The nitrocellulose strips were then washed in approximately 200 mL TBS for 10 minutes with shaking, followed by two similar washes in TBS- Tween and a final wash in TBS. The nitrocellulose strips were then incubated in the second (indicator) antibody- horseradish peroxidase conjugate solution at room temperature with gentle shaking. The second (indicator) antibody- enzyme conjugate was diluted according to the manufacturer's instructions (in TBS). The length of this incubation step had to be empirically

determined and varied from 4 hours to 12 hours depending on the batch of enzyme– labelled antibody. The washes were repeated as described before to remove any unbound second antibody. The nitrocellulose strips were then placed in the substrate solution and left without shaking, in the dark, for approximately 15 minutes. After colour development of the bands were complete, the reaction was terminated by immersion of the nitrocellulose strips in approximately 200 mL of distilled water. The individual lanes in the nitrocellulose strips were then read densitometrically at a wavelength of 600 nm. The nitrocellulose membrane was blotted dry between pieces of filter paper and stored in the dark.

Monoclonal antibody production

Reagents and equipment: The following were used in the production of monoclonal antibodies: Myeloma cells P3X63– Ag8.653, Balb/ cbyj mice, Dulbecco's Modified Eagles Medium (DMEM) (Gibco/ BRL, Burlington, Ontario), RPMI medium (Gibco/ BRL), fetal calf serum (Gibco/ BRL), horse serum (Gibco/ BRL), MEM non– essential amino acids (Gibco/ BRL), NCTC 109 medium (Gibco/ BRL), hypoxanthine– thymidine supplement (Gibco/ BRL), aminopterin (Gibco/ BRL), insulin (23 units per mg) (Sigma Chemical Company), sodium pyruvate (100 mM) (Hazleton Dutchland Inc., Denver USA), polyethylene glycol (PEG) 1000 (average MW 950– 1050) (Baker Chemical Company), sodium bicarbonate (Sigma Chemical Company), ammonium chloride (Sigma Chemical Company), trypan blue (Sigma Chemical Company), 2– mercaptoethanol (Sigma Chemical Company), dimethyl sulfoxide (DMSO) (Sigma Chemical Company), 2,6,10,14– tetramethylpentadecane (pristane) (Sigma Chemical Company) and antibiotics (penicillin and streptomycin) (Gibco/ BRL). A humidified CO_2 (5 to 7 percent) incubator (Johns Scientific)

equilibrated at 37° C, an inverted microscope (Olympus) a laminar flow hood (Johns Scientific) a 37° C water bath, dissection tools, a stop watch, a bench top clinical centrifuge (International Equipment Company, Mass. USA) a hemocytometer, sterile hypodermic syringes and needles (18 to 26 gauge) sterile tissue culture plasticware.

Preparation of HAT: Although some laboratories make up a complete hypoxanthine aminopterin thymidine (HAT) preparation, it was found (Campbell, 1984) that it is more practical to keep the aminopterin separate. This is for two reasons. Firstly, aminopterin is the most labile of the three components and has to be checked separately. In addition, since it is light sensitive, aminopterin has to be stored in the dark. Secondly, after the control myeloma cells in a fusion have died and the presence of aminopterin is no longer required in the medium, hypoxanthine and thymidine should still be added to the medium for a few days to facilitate medium dilution and cell adaptation. The hypoxanthine thymidine (HT) combination is available commercially (0.010 M hypoxanthine, 0.0016 M thymidine) (Gibco/ BRL) or, can be prepared in the following manner. One hundred and thirty six milligrams hypoxanthine and 39 mg thymidine are dissolved in 100 mL distilled water. The mixture is heated to 60 C to aid dissolution of the hypoxanthine, filter sterilized and stored at – 70 C. The aminopterin can be obtained as a 0.1 mM preparation (Flow Laboratories) or, is prepared as a 0.001 M solution using the following protocol. Eighteen milligrams aminopterin are dissolved in 50 mL 0.005 M NaOH. The solution is then neutralized with HCl, and made up to 100 mL with distilled water. A 10 fold dilution with distilled water yields a 0.1 mM solution, which is filter sterilized and stored in the dark at – 70° C. Four hundred microlitres of aminopterin solution is mixed with 1 mL HT solution and added to

every 100 mL of fusion medium.

Preparation of insulin: Ten milligrams of insulin (23 units per mg) was dissolved in 100 mL of distilled water, filter sterilized and stored at 4° C. Two hundred microlitres of this preparation was added to every 100 mL of fusion medium.

Preparation of 50 percent (W/ V) polyethylene glycol (PEG): Polyethylene glycol– 1000 (12.5 g) was sterilized by autoclaving and then allowed to cool to about 60° C. Twenty five mL of serum– free DMEM, previously heated to 37° C was then added and the mixture adjusted to pH 7.2 with sterile sodium bicarbonate (7.4 percent (W/ V)).

Preparation of antibiotics: The antibiotics used in this protocol were penicillin and streptomycin. These were obtained as a mixture (P/ S; penicillin base 10, 000 units/ ml, streptomycin base 10, 000 mcg/ ml) and added to the cloning medium at a concentration of 1 ml/ 100 mL medium.

Preparation of sera: The horse and fetal calf sera were heated at 56 C for 30 minutes to inactivate any complement that might be present. The sera were then centrifuged (700 *rpm*, 5 min) to remove any debris.

Preparation of peritoneal exudate feeder cells for the fusion procedure: A female BALB/ cbyj mouse was killed by cervical dislocation, sterilized for a few minutes in 70 percent ethanol, and placed on its back on a dissecting board. (Female mice were used because they have less fat). Fusion medium (5 ml) was then injected into the peritoneal cavity using a 26 gauge needle and the abdomen

of the mouse thoroughly massaged. A hypodermic syringe equipped with a 22 gauge needle was then used to recover as much fluid as possible from the peritoneal cavity. This fluid was then added directly to the fused cells preparation just before plating (Campbell, 1984).

Preparation of spleen cells for *in vitro* immunization, or for use as feeder cells during cell cloning procedures: Four to twelve week old BALB/ cbyj mice were killed by cervical dislocation and sterilized for a few minutes in 70 percent ethanol. The spleens were then removed and placed in a petri dish with 5 mL serum free DMEM. The fat and all other residual tissue were then trimmed away and the spleen transferred to another petri dish containing 5 to 8 ml serum free DMEM. A 10 mL hypodermic syringe equipped with a 21 guage needle was then used to poke several holes in the spleen. The medium was then gently flushed through the spleen until all of the cells were loosened from the organ capsule. A 10 mL syringe equipped with a 26 guage needle was then used to draw the cells up and down a few times, after which the cells were transferred to a sterile capped 10 mL tube. The cells were then centrifuged for five minutes at approximately 1000 *rpm*. The supernatant was then removed and the pellet tapped loose. The cells were then resuspended in 5 mL sterile, ice cold ammonium chloride (0.17 M) and incubated in an ice bath for 10 minutes. Five mL of ice cold DMEM plus 15 percent fetal calf serum was then added and the mixture centrifuged as before for 5 minutes. The supernatant was discarded and the pellet resuspended in DMEM plus 10 percent fetal calf serum. These spleen cells were now ready for *in vitro* immunization, or for use as feeder cells in the cloning procedure (Campbell, 1984).

Preparation of thymocyte–conditioned medium (TCM): This procedure was

performed according to the method of Campbell (1984). Young (12 to 14 day old) BALB/ cbyj mice were killed by cervical dislocation and sterilized in 70 percent ethanol. (It is important to use young animals as the thymus is involuted in older ones). The chest cavity of each animal was then opened, with care taken not to rupture the oesophagus or the trachea. The thymus was then removed and rinsed thoroughly in serum free RPMI medium. Using the plunger of a 10 mL disposable syringe as a pestle, the thymus was then pressed through a 50 mm mesh stainless steel screen into a 10 cm petri dish containing 5 mL serum free RPMI medium. The organ capsule was then discarded and the cells further dispersed by drawing them up and down twice with a 10 mL syringe equipped with a 21 guage needle. This procedure was then repeated with a 26 guage needle using moderate pressure. The cells were then washed twice in RPMI medium by centrifugation at 1000 *rpm* for 5 minutes, followed by resuspension with a 10 mL pipette. The cells were then counted using a haemocytometer and resuspended in a small flask at a density of 5×10^6 cells per ml, in RPMI medium supplemented with 5×10^{-5} M 2-mercaptoethanol and 20 percent fetal calf serum. The flask was then incubated at 37° C in a humidified CO_2 incubator for 2 to 4 days. The preparation was then centrifuged (1000 *rpm*, 5 min) and the supernatant sterilized with a 0.2 um filter. This preparation which is stable for several months was then frozen at – 70° C.

***In vivo* immunization**: This procedure was performed according to the method of Campbell (1984). The mice were injected intraperitoneally with approximately 50 ug of alpha toxoid per animal. After a period of 15 to 17 days the animals were boosted with the same amount of antigen via the same injection route. Seventy two hours later the animals were sacrificed by cervical dislocation and the spleens removed under sterile conditions for use in the fusion procedure.

In vitro **immunization**: This procedure was performed according to the method of Campbell (1984). The alpha toxoid was dissolved in PBS (1 mg/ ml) and sterilized under an ultra violet lamp for 30 minutes. The toxoid was then dissolved in DMEM + 10 percent heat inactivated fetal calf serum to a final concentration of approximately 50 ug per ml. The previously obtained spleen cells were then suspended in the toxoid mixture at a density of 5 x 10^6 cells per ml. An equal volume of thymocyte conditioned medium (TCM) was then added to the preparation and the mixture incubated in a small (20 ml) tissue culture flask for 4 to 5 days in a humidified CO_2 incubator. These cells were now ready for the fusion procedure.

Growth medium: The medium used for growth of the myeloma and established hybridoma cells was Dulbecco's Modified Eagle Medium (DMEM, Gibco). This medium was supplemented with glucose (4.5 g/ L) L– glutamine (0.484 g/ L) and 10 percent horse serum. The medium used to grow spleen cells was the same except that the horse serum was replaced by 10 percent fetal calf serum. The medium used for growth of thymus cells was RPMI supplemented with 20 percent fetal calf serum.

Fusion medium: The fusion medium was used to grow the freshly fused myeloma–immunized spleen cells. This medium consisted of the following ingredients: 350 mL DMEM supplemented with glucose (4.5 g/ L) and L– glutamine (0.484 g/ L), 100 mL horse serum, 50 mL NCTC 109, 5 mL sodium pyruvate, 5 mL MEM non essential amino acids, 7 mL HAT, [illegible] mL insulin

Freezing medium: The medium used for freezing cells (myelomas and hybridomas) consisted of 90 percent fetal calf or horse serum and 10 percent DMSO (V/ V).

Freezing procedure: The cells to be frozen were counted and then transferred from the tissue culture flasks into sterile centrifuge tubes. The cells were then centrifuged (1000 *rpm*, for 5 minutes) resuspended in freezing media at a concentration of around 2– 5 x 10^6 cells per ml, and then dispensed into freezing vials (1 mL per vial). The vials were packed in styrofoam, frozen at –20° C for 2 hours and finally transferred to the –70° C freezer.

Estimation of cell viability: One mL of the cell suspension was mixed with 1 mL of 0.2 percent trypan blue (in 0.15 percent NaCl (W/ V)). After thorough mixing the cells were counted in a hemocytometer and the percentage of dead cells (stained blue) estimated.

Fusion procedure: A frozen vial of myeloma cells was removed from the freezer and quickly thawed by shaking in a 56 C water bath. The cell suspension was transferred to a 50 mL tube and 10 mL of medium (DMEM supplemented with 10 percent horse serum) was added dropwise with gentle agitation. The cells were then centrifuged (1000 *rpm* for 5 min) and resuspended in 10 mL of medium. After estimation of viability, the cells were seeded into small flasks and grown until the density was in the range of 2 x 10^6 cells per ml. Cells in the logarithmic phase of growth were then harvested and their viability checked. Only cells with at least 98 percent viability were used in the fusion procedure. The total amount of myeloma cells needed for each fusion was 3 to 4 x 10^7.

The spleen cells that were immunized either *in vivo*, or *in vitro* were prepared

as previously described. The amount of spleen cells needed for each fusion was approximately five times the amount of myeloma cells obtained. The spleen and myeloma cell preparations were washed twice by centrifugation (1000 *rpm*, for 5 min) in serum free DMEM to remove all traces of serum. Both cell preparations were then counted and then combined at a ratio of 5 spleen cells for every myeloma cell. The mixture of cells was then centrifuged (1000 *rpm*, for 5 min) and all of the supernatant removed to obtain a totally dry pellet. One mL of PEG (50 percent in DMEM, heated to 37° C) was then added to the intact pellet over a 1 minute period while stirring gently with the pipette tip. The gentle stirring with the pipette tip was continued for another minute, after which the preparation was allowed to sit undisturbed for a third minute. Five mL of serum free DMEM was then added at a rate of 1 mL per minute with constant gentle swirling. An additional 4 mL of serum free DMEM was then added all at once. The mixture was then centrifuged at 700 *rpm* for 5 minutes and the supernatant discarded. Fresh serum free DMEM was then added and the centrifugation repeated. The cells were then gently resuspended in fusion medium. Feeder cells from the peritoneal exudate were now obtained and added to the fused cell preparation. The cells were then plated 0.2 mL per well in 96 well flat botommed plates, at a concentration of 5×10^4 to 10^6 cells per well. The covered plates were then incubated at 37° C in a humidified 5 percent CO_2 incubator. These plates were allowed to sit undisturbed for 2 to 5 days after which they were examined for the presence of foci. Controls in this procedure consisted of spleen and myeloma cells seeded in separate wells.

Post- fusion care of hybridomas: At this stage it is important to note that every tissue culture operation performed on the fused cells, increases the risk of

contamination and may also disturb the emerging colonies. Consequently, the cells should be fed as infrequently as possible. On the other hand, to increase the chromosome stability, all colonies should be assayed for antibody production and the positive ones cloned as early as possible. In addition, it should also be noted that aminopterin inhibits many normal cell functions and should be removed from the feeding medium as early as possible. The time at which aminopterin is discontinued can best be determined by monitoring the myeloma control wells. As soon as these control cells are dead the aminopterin should be removed from the feeding medium. Hypoxanthine and thymidine however, should still be supplied since the emerging hybridoma cells may need time to adapt to the *de novo* pathways of purine and pyrimidine biosynthesis.

The wells were inspected after 5 days to determine if there were any live cells in the test wells and if all the myeloma control cells were dead. At this stage, if the control myeloma cells still looked healthy, 100 ul complete fusion medium was added to all the wells, after initially removing 100 ul of the spent medium from each well. This was accomplished using an eight channel multi– pipette (Finnpipette, Fisher Scientific Ltd.) with care taken not to disturb any of the emerging colonies. The wells were then monitored on a daily basis and after 7 to 10 days the cells were fed 100 ul medium as outlined above. If the control myeloma cells were still alive, the feeding medium contained aminopterin. If the control cells were dead aminopterin was omitted. Between 10 and 14 days, visible colonies appeared. These were visible with the naked eye when looking from below at the plate held up to the light. Some of these colonies grew faster than others, and this resulted in a change in the pH of the medium in the wells. This pH change resulted in the colour of the medium changing fron deep pink to yellow. These fast– growing colonies were fed and the media that were removed were

saved for analysis of their antibody content. By day 18 all the colonies were large enough to be tested for antibody production. This was accomplished using an ELISA.

Cloning by limiting dilution: Colonies that were deemed positive by the ELISA for anti- alpha toxin production, were selected for cloning. The medium from wells that contained individual anti- alpha toxin producing colonies was discarded and the colonies aspirated into 1 mL of fresh DMEM supplemented with 10 percent horse serum and antibiotics. The cells were counted and a small number (approximately 10^3) retained for cloning. The remaining cells were transferred to a single well in a 24- well plate, fed, and incubated at 37° C. Five 96- well plates were next seeded with freshly prepared spleen feeder cells at a fairly high concentration (approximately 10^6 cells per ml). (This concentration was found not to be critical). The cells to be cloned were then suspended in DMEM supplemented with 10 percent horse serum at a concentration of 10 cells per ml. This cell suspension was then added (0.1 mL per well) to two of the 96- well plates seeded with the feeder cells. The remaining hybridoma cells were then diluted to a concentration of 5 cells per mL and similarly plated into the remaining 96- well plates. These plates were then incubated for 7 to 14 days until visible clones appeared. These clones were assayed for anti- alpha toxin production with the ELISA, and the positive ones subcloned. This cloning procedure was repeated once more to ensure chromosome stability of the cells. The anti- alpha toxin producing subclones and the uncloned parent culture were sequentially expanded into 24- well, 6- well and 25 cm^2 flasks for freezing, and in the case of the subclones, for ascites fluid production.

Production of ascites fluid: The hybridomas were grown in the peritoneal cavity of animals of the same strain as the tumor cell line donor and the spleen cell donor. Monoclonal antibodies were secreted into the ascites fluid formed within this cavity.

BALB/ cbyj mice were injected intra– peritoneally with 1 mL pristane (2,6,10,14– tetramethyl– pentadecane) 7 to 14 days prior to injection with the hybridoma cells. When ready, the subcloned cells were centrifuged (1000 *rpm*, for 5 minutes) resuspended in fresh medium (DMEM supplemented with 10 percent horse serum) and injected intraperitoneally (approximately 10^7 cells per mouse) into pristane– primed mice. The mice were left undisturbed for about 5 days, after which they were monitored daily for distension of the abdomen. Ten to fourteen days after injection, the mice with greatly distended abdomens were sacrificed by cervical dislocation. A small patch of the abdominal skin was then removed and a small incision was made in the peritoneum with a pair of scissors. A pasteur pipette was then used to aspirate the ascites fluid. This was repeated until no more fluid was obtained from the animal. The ascites fluid was then centrifuged and the supernatant containing the monoclonal antibody was stored at –20° C. The cells were resuspended in PBS (without azide) and reinjected into pristane–primed mice. This procedure was repeated for a total of three passages. After the third passage the cells were discarded. A new cycle was then started with fresh hybridoma cells.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Reagents: All electrophoresis reagents were of electrophoresis purity grade (Bio Rad Laboratories, California, USA). Low molecular weight standards (Bio Rad

Laboratories) were included in every electrophoretic run.

Sample buffer: A pre– solubilization stock buffer consisting of 0.040 M Tris base, 0.020 M sodium acetate and 0.002 M disodium EDTA in distilled water, adjusted to pH 7.4 with glacial acetic acid, was prepared and stored in a glass container at 4° C. The final solubilization buffer was made by the addition of 2 percent (W/ V) SDS, 5 percent (W/ V) dithioerythritol and 12 percent (W/ V) sucrose to the pre– solubilization buffer, just prior to use.

Electrode buffer: The electrode solution in the upper (cathode) and lower (anode) buffer compartments consisted of 0.025 M Tris, 0.188 M glycine and 0.0035 M SDS in distilled water.

Sample preparation: Samples to be separated were prepared by combining the protein preparation (dissolved in distilled water, approximately 50 ug per ml) with the solubilization buffer at a ratio of 1 part protein to 5 parts buffer. Bromphenol blue (0.05 percent, 10 ul per sample) was then added and the sample vortexed for 20 seconds. Samples were then placed in a boiling water bath for 6 minutes just before application (40 ul per well) to the previously prepared SDS– PAGE gel.

Gel preparation: The gel system used was a discontinuous one dimensional SDS– PAGE procedure similar to the second dimension of the 2D technique described by O' Farrell (1975).

Separating gel: The separating gel consisted of 12 percent (W/ V) acrylamide/ bis– acrylamide stock (30 percent T, 6.3 percent C) 0.375 M Tris (pH 8.8) 0.1

percent (W/ V) SDS, 0.5 percent (W/ V) fresh ammonium persulfate and 0.5 percent (V/ V) N,N,N',N' – tetramethylethylenediamine (TEMED). These ingredients were combined, degassed with a vacuum pump and poured into a slab gel mould (15.0 cm x 8.0 cm x 0.15 cm). The gel was overlayed with distilled water and allowed to polymerize undisturbed, for 3 hours at room temperature. The top of the gel was then rinsed with distilled water and allowed to dry.

Stacking gel: A stacking gel (15.0 cm x 2.0 cm x 0.15 cm) consisting of 4 percent acrylamide/ bis– acrylamide stock, 0.125 M Tris (pH 6.8) 0.1 percent SDS, 0.05 percent fresh ammonium persulfate and 0.1 percent TEMED, was then degassed and overlayed unto the separating gel. A teflon comb was used to form sample application wells within the stacking gel. The gel was allowed to polymerize for two hours and then was stored at 4° C overnight. Just before use, the teflon comb was removed and the wells washed with distilled water.

Run conditions: A constant current of 12 milliamperes was applied for 5 to 6 hours, or, until the bromphenol blue tracking dye reached the bottom of the separating gel. The gel was constantly cooled by running cold tap water aided by constant stirring of the lower electrode solution.

Digestion of Alpha Toxin with Cyanogen Bromide

The cyanogen bromide digestion was performed according to the procedure of Gross and Witkop (1961). Alpha toxin was dialyzed extensively against distilled water, denatured by boiling for 3 minutes and lyophilized. The toxin was then dissolved in 70 percent formic acid at a concentration of 5 mg per mL in a glass

stoppered bottle. A 100 fold molar excess over methionine residues of crystalline cyanogen bromide was then added and the solution thoroughly flushed with nitrogen. The reaction was then allowed to proceed with constant shaking in the dark at room temperature for 48 hours. The reaction was stopped by diluting the reaction mixture with 15 volumes of distilled water. The resulting solution was then shell frozen and lyophilized. The lyophilized material was then redissolved in distilled water and relyophilized for complete removal of of the acid and by-products. The final product was reconstituted in distilled water.

Digestion of Alpha Toxin with Trypsin

The digestion with trypsin was performed according to the procedure of Allen (1981). The alpha toxin was dialyzed extensively against distilled water, denatured by boiling for 3 minutes and lyophilized. The toxin was then dissolved in a volatile buffer (0.1 M ammonium carbonate supplemented with 0.1 mM calcium chloride) and the pH of the solution adjusted to 8 TO 8.5 with ammonium hydroxide. TPCK treated trypsin (Type XIII, 10, 000 to 13, 000 BAEE units per mg protein) (Sigma) was then added as a solution (10 mg per mL in 0.1 mM HCl) at a concentration of 5 percent by weight of the protein. Samples were removed at various times and the reaction stopped by the addition of soybean trypsin inhibitor.

Protein Assays

Protein concentrations were determined according to the method of Bradford (1976).

Buffers

All buffers not specifically mentioned in the text were prepared according to Chase (1968). Reference standard buffer solutions were obtained from Fisher Scientific Co. (New Jersey, USA).

Chemicals

All chemicals not specifically mentioned were reagent grade.

CHAPTER ONE

Development of an enzyme– linked immunosorbent assay (ELISA) for detection of *Staphylococcus aureus* alpha toxin

Introduction

Staphylococcal alpha toxin is generally measured by the hemolytic titration assay (Bernheimer and Schwartz, 1963; Dalen, 1976; Fussle *et al.*, 1981; Lo and Fackrell, 1980; Wiseman and Caird, 1970; Wiseman and Caird, 1972). However, this assay has several disadvantages, such as variability (Arbuthnott, 1970; Bernheimer, 1965; Bernheimer and Schwartz, 1963; Cooper *et al.*, 1966) and lack of standardization (Lominski *et al.*, 1963; Robinson and Thatcher, 1963) which limit its use as a quantitative method. The use of enzyme– labelled antibodies in the form of the enzyme– linked immunosorbent assay (ELISA) was pioneered by Engvall and Perlmann (1971; 1972) and Van Weemen and Schuurs (1971; 1972). Since its introduction, ELISA techniques have gained wide acceptance (Akerlund *et al.*, 1977; Al– Bassam *et al.*, 1978; Berdal *et al.*, 1981; Clark and Adams, 1977; Crosson *et al.*, 1978; Ruitenberg and van Knapen, 1977; Stevens and Tsiantos, 1979; van Knapen and Panggabean, 1977; Walls *et al.*, 1977; Yolken *et al.*, 1977) over the equally sensitive radioimmunoassay, for a number of reasons as reviewed by Wisdom (1976) and O' Sullivan *et al.*, (1979).

This chapter describes the development of a double– antibody sandwich (Voller *et al.*, 1976; Voller *et al.*, 1979) ELISA for the detection of the alpha toxin of *Staphylococcus aureus* Wood 46. This form of ELISA overcomes the limitations of the hemolytic titration assay.

Results

Antibody coating of the polystyrene surface

An Elisa was performed in which four different concentration of antitoxin (32 replicates) were used to coat the wells of the microtitre plate and the polystyrene–coated beads. An undiluted *S. aureus* culture supernatant, an antibody– enzyme conjugate dilution of $\log_{10}2$, and a substrate concentration of 1 mg/ ml were used. The concentration of immunoglobulin that gave an optimal coating was 0.2 ug per well (figure 1. 1) after 4 to 6 hours at 37° C, or, overnight at 4° C.

When polystyrene– coated steel beads were used as the adsorbent, the initial incubation stage could be eliminated by storage of the beads at 4° C in an immunoglobulin solution of approximately 1 ug/ ml. Then, immunoglobulin– coated beads were available for immediate use.

Antigen

The binding of alpha toxin to the adsorbed immunoglobulin was examined at three different temperatures (32 replicates). Assays were set up with an undiluted *S. aureus* culture supernatant as the antigen source, an antitoxin concentration of 2.0 ug per well, an antibody– enzyme conjugate dilution of $\log_{10}$ 2, and a substrate concentration of 1 mg/ ml. It was found that of the three temperatures examined, maximal binding of alpha toxin occured at 27° C with a mean absorbance value of 0.342 and a coefficient of variation of 0.037 (table 1. 1). Maximum absorption of the toxin was obtained after overnight incubation.

Antibody– enzyme conjugate

Generally, a dilution between $\log_{10}$ 2 and $\log_{10}$ 3 yielded high sensitivity when the plates were incubated at 37° C for 4 to 8 hours (figure 1. 2). The conjugate was found to be stable for a period of several months when stored at –20° C.

Substrate

In an effort to determine a convenient time and incubation temperature for the substrate, assays were performed in which all components were in excess. Three different temperatures were examined with the reactions stopped at various times with 3 M NaOH. Absorbance values increased linearly over the initial 60 minutes to a value of approximately 0.9 absorbance units (corrected for the control) after which a plateau was obtained (figure 1. 3). The plateau effect was due to the limitations of the spectrophotometer that was used to measure the absorbance (figure 1. 4). Since high sensitivity was obtained at 37° C, this substrate incubation temperature was chosen for subsequent tests which were stopped at 60 minutes. Once an optimal time at a suitable temperature was established, three different substrate buffers (diethanolamine, Tris and borate) at different pH values were examined for their effect on the sensitivity of the assay. Borate buffer inhibited the assay. However, with Tris and diethanolamine buffers, although a low degree of sensitivity was observed between pH 7 and 8, there was a rapid increase in absorbance between pH 8.5 and 9, with maximum sensitivity achieved at pH values greater than 9.5 (figure 1. 5). Once again, the plateau effect that was observed in the absorbance readings, was due to the limitations of the spectrophotometer. In subsequent tests, Tris buffer was used because of its greater stability.

Comparison of the ELISA and the hemolytic titration assay

To compare the sensitivity of the ELISA with that of the hemolytic titration assay, 20 different concentrations (4 replicates) of alpha toxin were simultaneously tested with both assays. The ELISA was set up with three constant components: the wells coated with antibody, the antibody– enzyme conjugate, and the substrate, all in excess. The hemolytic titration assay was performed as previously described (Lo and Fackrell, 1980). In the hemolytic titration assay, lysis could not be detected beyond a $\log_2$ 7 dilution. On the other hand, in the ELISA, the presence of toxin was detectable even at a $\log_2$ 17 dilution (figure 1. 6). The ELISA is thus 500 to 1, 000 times more sensitive than the hemolytic titration assay.

Effect of protein A on the sensitivity of the ELISA

Staphylococcus aureus Wood 46 produces negligible amounts of IgG– binding protein A as assayed for by single radial immunodiffusion (data not shown). These results reiterate those of Forsgren (1970) Forsgren *et al.*, (1976) and Lachowicz *et al.*, (1976). Other isolates of *Staphylococcus aureus*, however, release extracellular protein A in the range of, 2.8 to 720 ng/ ml of culture broth. Protein A, when present in the culture supernatant, may cross– link the immobilized antibody with the detecting antibody– enzyme conjugate, thus leading to false positive results.

An ELISA was performed to determine the degree of the effect of protein A on the sensitivity of the assay. A supernatant of a culture of *Staphylococcus aureus* Wood 46, that was supplemented with excess protein A (1 ug/ ml) was used as the test sample (20 replicates). The unsupplemented supernatant served as the control (20 replicates). The results (table 1. 2) indicate that a 12 percent

higher absorbance value was obtained in the protein A supplemented samples. A P value of 0.0327 indicates that the difference between the control and the test absorbance values is significant. These results indicate that protein A in the samples does cross- link the adsorbed and immobilizing antibodies, thus leading to false positive values.

Figure 1. 1

Effect of different coating concentrations of antitoxin on the sensitivity of the ELISA. An undiluted *S. aureus* Wood 46 culture supernatant was used as the alpha toxin source. An antibody–enzyme conjugate dilution of $\log_{10}$ 2, and a substrate (PNPP) concentration of 1 mg/ ml were used. The substrate (PNPP) was hydrolyzed to yield PNP which is detected at 405 nm. The spectrophotometer used has a maximum absorbance reading of 2.0, and the antigen– negative control value was 0.2 absorbance units. The absorbance values for the three highest antitoxin concentrations, all of which were > 2 (calculated from a 1: 2 dilution of the well contents), are therefore expressed as 1.8 units.

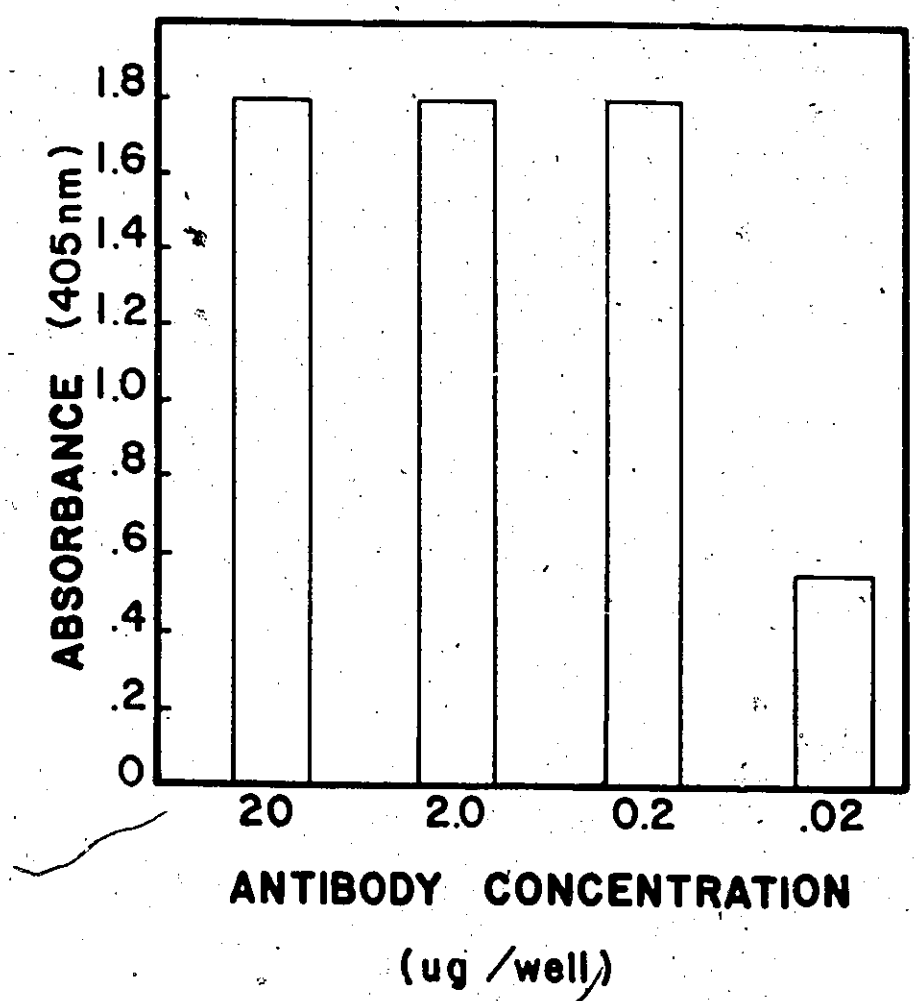

ABSORBANCE (405nm)
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
20
2.0
0.2
.02
ANTIBODY CONCENTRATION
(ug /well)

Figure 1. 2

Effect of different dilutions of the antitoxin– alkaline phosphatase conjugate on the sensitivity of the ELISA. The wells of the microtitre plate were coated with antitoxin at a concentration of 0.2 ug per well. An undiluted *S. aureus* culture supernatant was used as the alpha toxin source. The substrate (PNPP) was used at a concentration of 1 mg per ml. The substrate (PNPP) was hydrolyzed to yield PNP which is detected at 405 nm. The spectrophotometer used has a maximum absorbance reading of 2.0, and the antigen– negative control value was 0.2 absorbance units. The absorbance values for the two lowest antibody– conjugate dilutions, both of which were > 2 (calculated from a 1: 2 dilution of the well contents) are therefore expressed as 1.8 absorbance units.

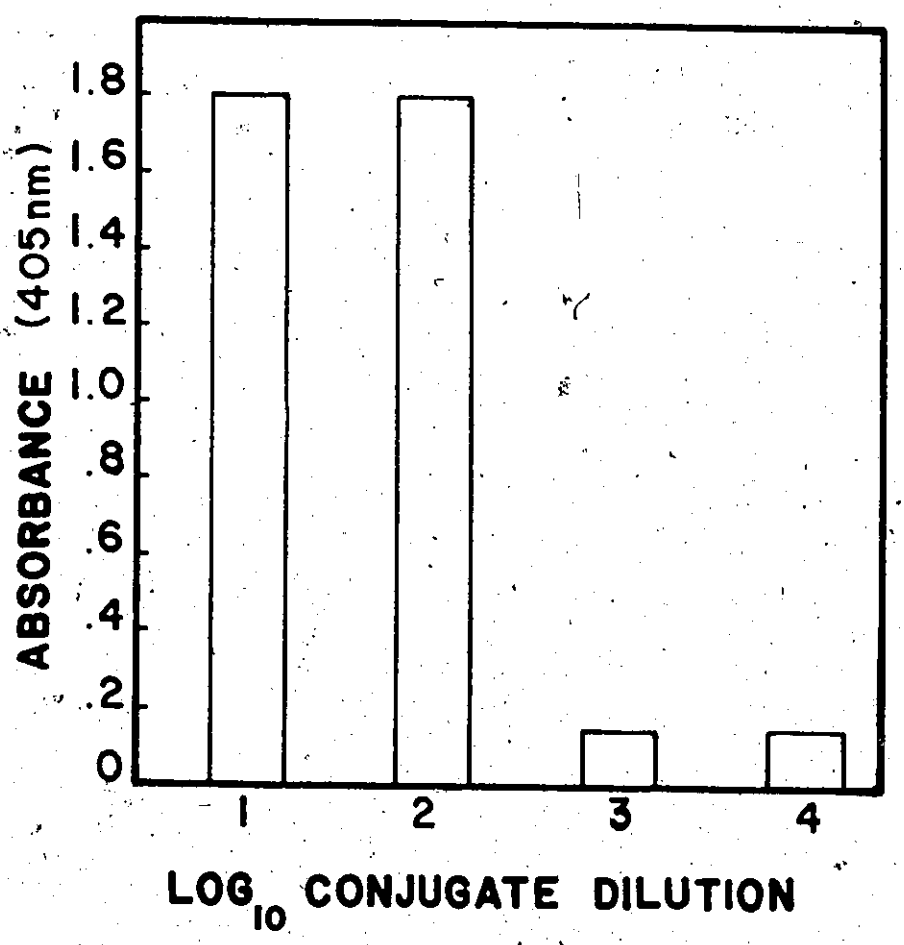

ABSORBANCE (405nm)
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
1
2
3
4
LOG10 CONJUGATE DILUTION

Figure 1. 3

Effect of different substrate incubation times and temperatures on the sensitivity of the ELISA. Symbols: ●, 4σ C; o, 27σ C; ■, 37σ C. The wells of the microtitre plate were coated with antitoxin at a concentration of 0.2 ug per well. An undiluted *S. aureus* culture supernatant was used as the alpha toxin source. The antibody–enzyme conjugate was diluted $\log_{10}$ 2, and the substrate concentration was 1 mg/ ml.

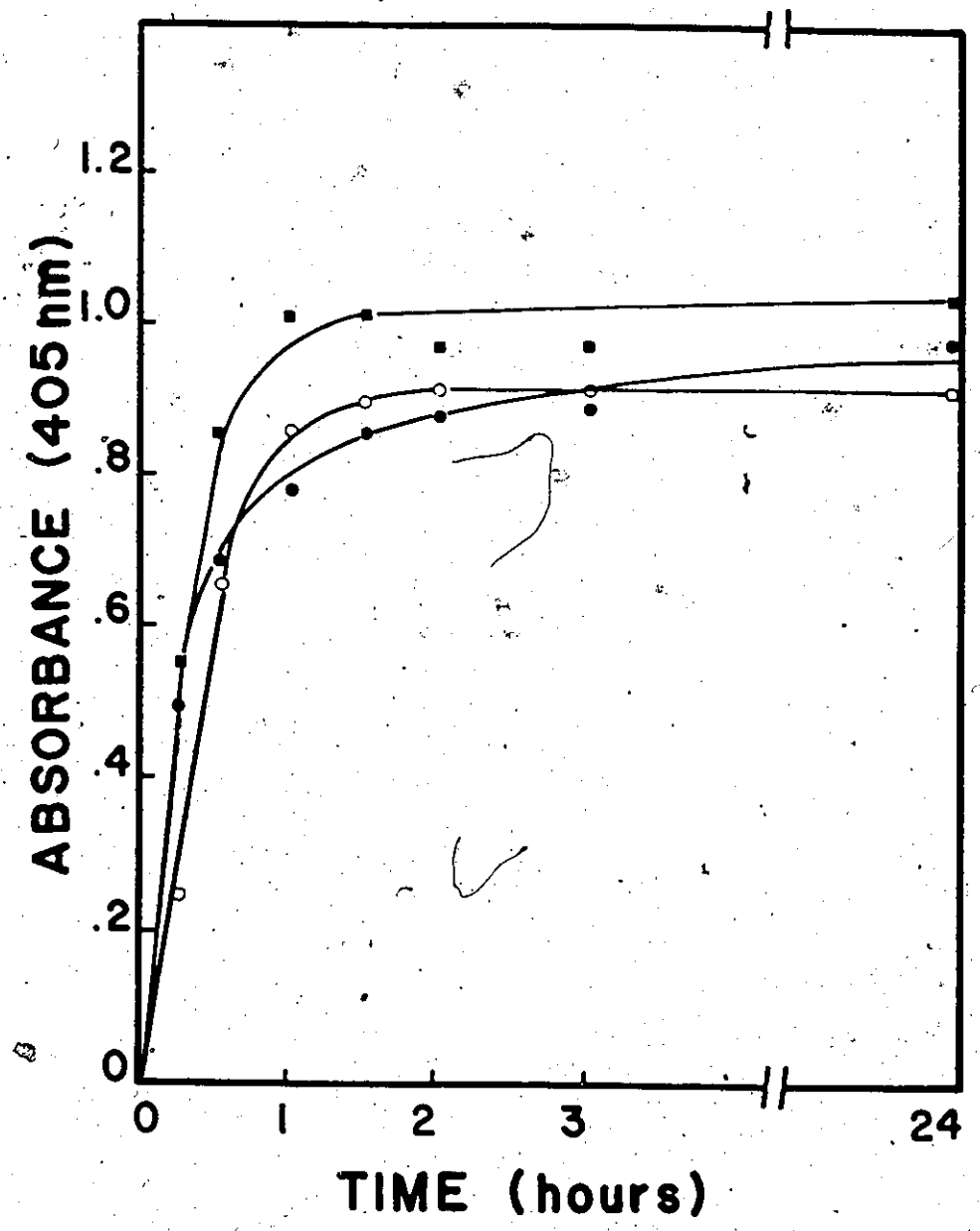

ABSORBANCE (405 nm)
1.2
1.0
.8
.6
.4
.2
0
0
1
2
3
24
TIME (hours)

Figure 1. 4

This graph shows the measurement of different concentrations of p– nitro phenol (PNP) at 405 nm, using the Perkin– Elmer double beam spectrophotometer. This experiment, was designed to measure the linearity of the machine response over a range of PNP concentrations at 405 nm. In this experiment, p– nitro phenyl phosphate (PNPP) (1 mg/ ml) was hydrolyzed with antibody– alkaline phosphatase conjugate for 12 hours at 37^{o} C. The product of this hydrolysis (PNP), was then serially diluted log $_2$ in substrate buffer and the absorbances read at 405 nm (1 cm light path).

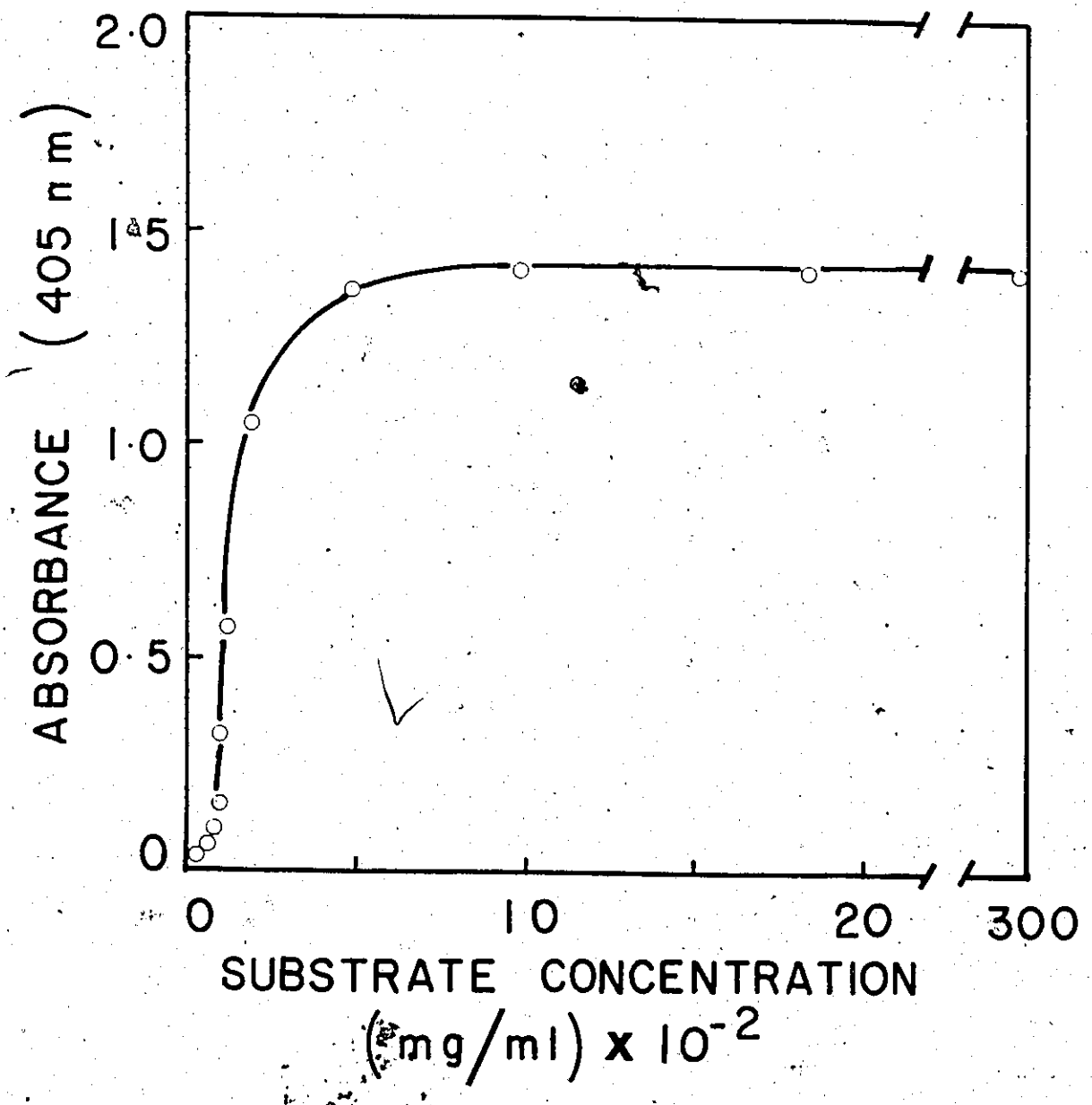

2·0
1·5
1·0
0·5
0
ABSORBANCE (405 nm)
0
10
20
300
SUBSTRATE CONCENTRATION
(mg/ml) x 10⁻²

Figure 1. 5

Effect of three buffers at different pH values on the sensitivity of the ELISA. Symbols: ●, diethanolamine; o, Tris; and □, borate. The wells of the microtitre plate were coated with antitoxin at a concentration of 0.2 ug per ml. An undiluted *S. aureus* culture supernatant was used as the alpha toxin source. The antibody–enzyme conjugate was diluted $\log_{10}$ 2, and the substrate concentration was 1 mg/ ml.

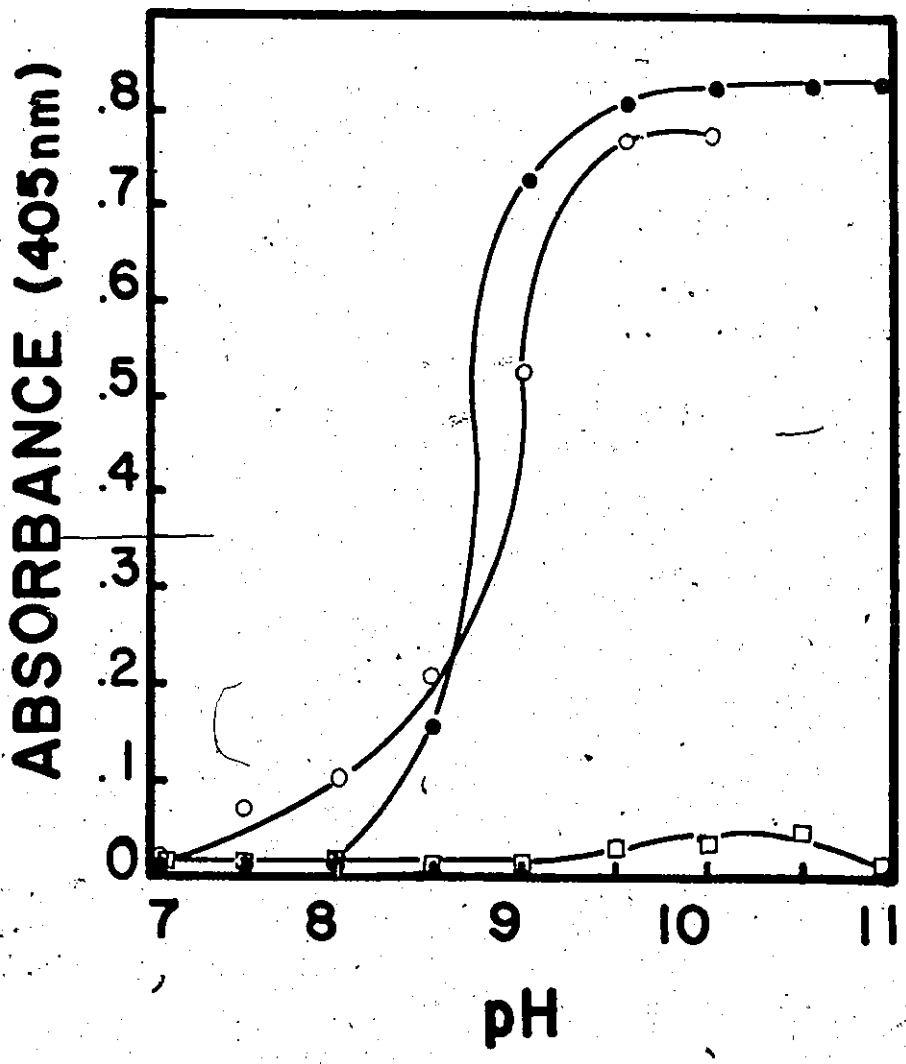

ABSORBANCE (405nm)
0
.1
.2
.3
.4
.5
.6
.7
.8
7
8
9
10
11
pH

Figure 1. 6

Comparison of the relative sensitivities of the ELISA (●, 405 nm) and the hemolytic titration assay (o, 541 nm) in measurement of alpha toxin. Both assays were performed as described in Materials and Methods on identical toxin samples.

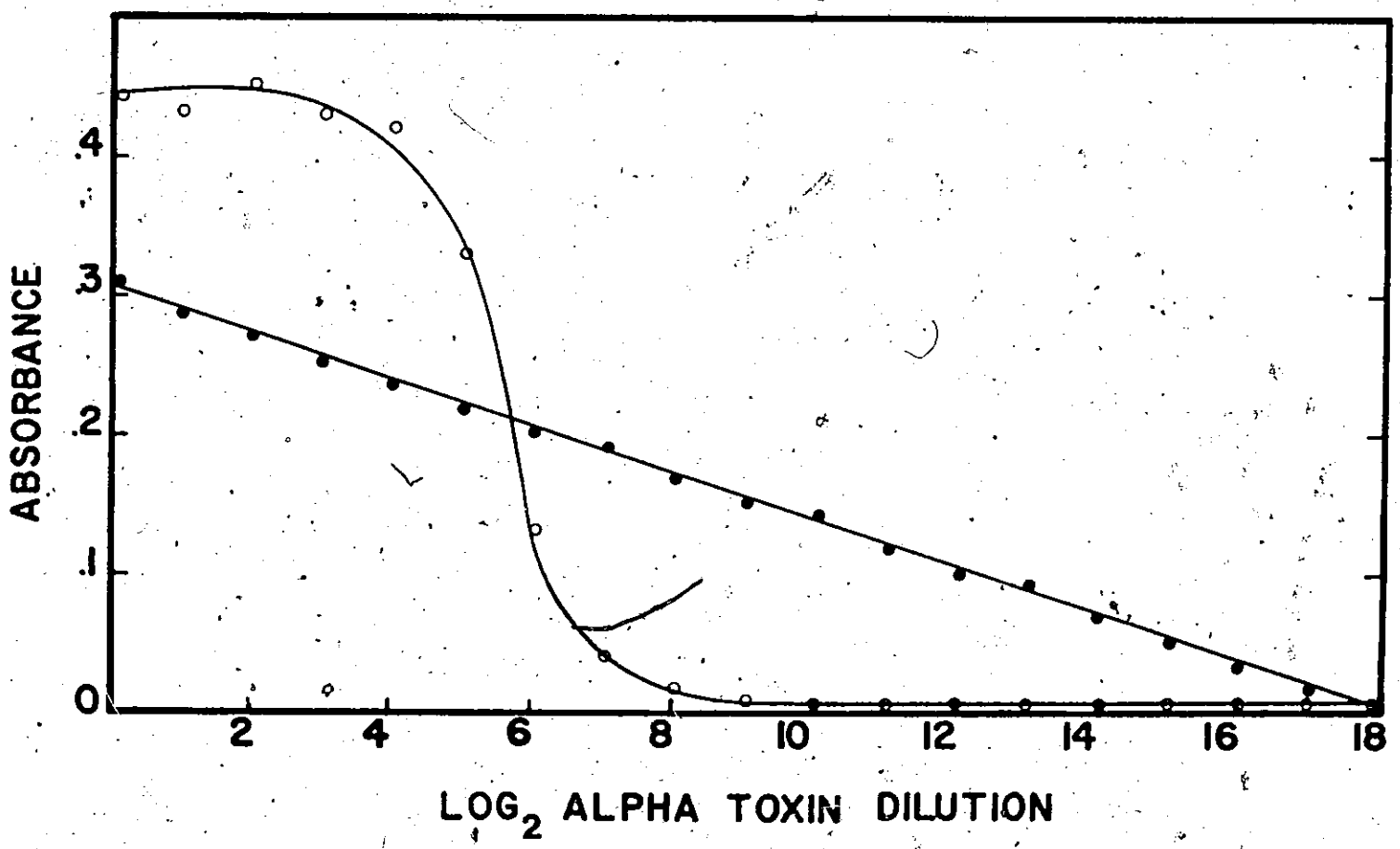

ABSORBANCE
0
.1
.2
.3
.4
2
4
6
8
10
12
14
16
18
LOG2 ALPHA TOXIN DILUTION

Table 1. 1

Effects of different temperatures for incubation of alpha toxin on ELISA sensitivity

Incubation Temperature (C)	Mean Absorbance Value (n = 32)	Coefficient of Variation
4	0.0528	0.803
27	0.3420	0.037
37	0.1560	0.639

The wells of the microtitre plate were coated with antitoxin at a concentration of 0.2 ug per well. An undiluted *S. aureus* culture supernatant was used as the toxin source. The antibody– enzyme conjugate was diluted $\log_{10}$ 2, and the substrate concentration was 1 mg/ ml.

Table 1.2

Effect of protein A on the ELISA sensitivity

Antigen	Mean absorbance value (n = 20)
S. aureus culture supernatant	0.602
S. aureus culture supernatant supplemented with protein A (1 ug/ml)	0.693

The wells of the microtitre plate were coated with antitoxin at a concentration of 0.2 ug per well. The antibody- enzyme conjugate was diluted $\log_{10}$ 2, and the substrate concentration was 1 mg/ml. A P value of 0.0327 indicates that these two values are significantly different

Discussion

Hemolytic titration is the most convenient and sensitive bioassay available for the measurement of alpha toxin (Arbuthnott, 1970) whether it is read as 50 percent lysis visually (Bernheimer and Schwartz, 1963; Dalen, 1976; Fussle *et al.*, 1981; Wiseman and Caird, 1970) or 50 percent lysis spectrophotometrically (Lo and Fackrell, 1980; Wiseman and Caird, 1972). However, there are associated with this bioassay, several inherent disadvantages which can be overcome by substitution with the ELISA. Firstly, there is no internationally accepted hemolytic unit that is defined in terms of a unit measurement of the toxin protein. Values of this unit range from 0.008 ug (Lominski *et al.*, 1963) to 0.5 ug (Robinson and Thatcher, 1963). A direct comparison of the hemolytic assay of lominski *et al.* (1963) with that of Bernheimer and Schwartz (1963) has revealed a fivefold difference in the sensitivity of the assays. Furthermore, several researchers (Bernheimer, 1965; Bernheimer and Schwartz, 1963; Cooper *et al.*, 1966; Timmerman, 1937) have found that the sensitivity of erythrocytes from different rabbits may vary also up to fivefold with the same batch of toxin. Such variability, combined with the wide variety of experimental techniques employed by different workers has made the task of standardization of the hemolytic titration assay a difficult one. In contrast, the ELISA can be made highly quantitative since the binding of antibody– enzyme conjugate is proportional to the amount of alpha toxin. Quantitation and subsequent standardization can then be achieved by correlating toxin concentration with enzyme activity under specified conditions.

Second, it has been demonstrated, that in any alpha toxin preparation, there is always a percentage of toxoid (Arbuthnott *et al.*, 1973) which binds to the same

receptors on the rabbit erythrocyte membrane as toxin, but does not cause lysis (Barei and Fackrell, 1979). Thus, in any preparation, there is always a dynamic competition between toxin and toxoid for membrane binding sites. The amount of lysis that results from this competition is a reflection of the ratio of toxin to toxoid in the preparation rather than the total amount of toxin. This phenomenon further contributes to the inaccuracy of the hemolytic titration assay. The ELISA on the other hand, does not discriminate between the binding of toxin and toxoid to the antitoxin. The titre of alpha toxin as measured by hemolytic titration varies from day to day (unpublished data) a phenomenon which might reflect toxin– toxoid interconversion. This instability does not appear in the ELISA once procedures are carefully duplicated.

Finally, as demonstrated in figure 1. 6, the ELISA is 500 to 1, 000 times more sensitive than the hemolytic titration assay. Furthermore, in the ELISA, absorbance varies linearly with concentration, and the apparent threshold effect as seen in the hemolytic titration assay is not displayed. The ELISA is seen to be a relatively simple, safe, economical, and quantitative alternative for the detection of staphylococcal alpha toxin.

One possible inconvenience with this alpha toxin ELISA, is the interference of IgG– binding protein A when it is also present in the supernatant along with the alpha toxin. *Staphylococcus aureus* Wood 46 produces negligible amounts of protein A (Forsgren, 1970; Forsgren *et al.*, 1976; Lachowicz *et al.*, 1976) and is therefore free of this interference. On the other hand, other isolates of *Staphylococcus aureus* which release high levels of protein A, cannot be readily assayed for alpha toxin with this assay. However, Berdal *et al.*, (1981) have resolved the problem, by suggesting that culture fluids with excessive amounts of protein A can be depleted simply by adsorption with IgG– coated beads.

CHAPTER TWO

Fragmentation analysis of staphylococcal alpha toxin
Isolation and identification of a binding fragment

Introduction

Staphylococcal alpha toxin is an exoprotein which exhibits biological properties such as lethal, dermonecrotic, membrane– damaging and hemolytic activities (Freer and Arbuthnott, 1976, 1983; Jeljaszewitz, 1972; Jeljaszewitz *et al.*, 1978; Rogolsky, 1979; Wiseman, 1975). This protein is composed of one polypeptide chain (Arbuthnott, 1970; Bernheimer *et al.*, 1963; Lominski *et al.* 1963; Six *et al.*, 1973a, b) with a molecular weight of approximately 34, 000 (Freer and Arbuthnott, 1983; Gray and Kehoe, 1984; Thelestam, 1983). Several amino acid analyses have been performed on this toxin and the results are in reasonable aggreement (Bernheimer and Schwartz, 1963; Coulter, 1966; Fackrell and Wiseman, 1976a; Kato and Watanabe, 1980; Six and Harshman, 1973a; Watanabe and Kato, 1978; Wiseman and Caird, 1970). The complete DNA sequence of a cloned alpha toxin gene has also been determined and the amino acid composition of the protein predicted from the DNA sequence, is very similar to that obtained with purified alpha toxin (Gray and Kehoe, 1984).

Although the *in vivo* target organ is not yet known, the large diversity of cells which is affected by this toxin, suggests that susceptible cells possess a common target site. Since the cell membrane constitutes the primary contact site with the toxin, studies have focussed on the interaction between the toxin and membranes. This interaction has been extensively studied in hemolytic systems (Barei and Fackrell, 1979; Bernheimer *et al.* 1972; Bhakdi *et al.*, 1984; Cassidy and Harshman,

1973, 1976a, b; Freer *et al* 1968, 1973; Kaplan, 1972; Klainer *et al*, 1964) that employed rabbit erythrocytes which exhibit the greatest sensitivity to alpha toxin of any cell type tested. Maharaj and Fackrell (1980) have shown that a receptor for alpha toxin is present in band three of the rabbit erythrocyte. More reaelt umrk (Simpson, 1986, MSc Thesis) has further identified the receptor to be the carbohydrate moiety of band 3. Alpha toxin– mediated damage to other cell types has also been reported. Artenstein *et al*, (1963) demonstrated the release of ^{35}S methionine from pre– labelled rabbit kidney and human ammnion cells after treatment with alpha toxin. Thelestam's group (Thelestam, 1983a, b; Thelestam *et al*, 1973; Thelestam and Mollby, 1975a, b, 1976) have also reported on alpha toxin–induced membrane damage to cultured mammalian cells and have also postulated the presence of a receptor on mouse adrenocortical (Y1) tumor cells (Thelestam, 1983b).

Studies have suggested that separate regions on the alpha toxin molecule are responsible for receptor binding and membrane damage, in both the rabbit erythrocyte (Barei and Fackrell, 1979; Cassidy and Harshman, 1976a, b) and the Y1 tumor cell (Thelestam and Blomqvist, 1984). Blomqvist and Thelestam (1986) Kato and Watanabe (1980) and Watanabe and Kato (1978) using fragmentation analysis have also demonstrated that separate regions on the toxin molecule are responsible for binding, lethality and hemolysis. Harshman *et al*, (1986) in a structure–function analysis of alpha toxin, have suggested that the C– terminus segment does not interfere with binding to the specific receptor.

Lo and Fackrell (1979) have produced antibodies that prevent binding of alpha toxin to the membrane (anti– binding antibodies) and antibodies that prevent membrane damage caused by toxin already bound to the membrane (indirect hemagglutinating antibodies). Lo (1984, PhD Dissertation) has suggested that alpha

toxin possesses at least two anti- binding antigenic determinants and one indirect hemagglutinating antigenic determinant.

In this chapter, we report the isolation of immunologically reactive fragments of alpha toxin th't were generated by limited proteolysis and cyanogen bromide degradation. These fragments were recognized by whole antitoxin as well by purified preparations of indirect hemagglutinating and antibinding antibodies. Furthermore, a cyanogen bromide generated fragment of approximately 9, 000 daltons that binds to the membrane receptor, but does not cause hemolysis is also reported.

Results

Competitive enzyme– linked immunoassay (CELIA) for detection of immunologically reactive fragments of alpha toxin.

This assay was designed as a screening mechanism to detect fragments of alpha toxin that contain a single antigenic determinant. The agent chosen for the preliminary fragmentation studies was trypsin at a concentration of 1 percent by estimated weight of alpha toxin. An undiluted *Staphylococcus aureus* culture supernatant was used as the alpha toxin source. The use of alpha toxin in this crude form allowed the assay to be developed under conditions that allowed maximum possible interference. The concentration of alpha toxin was estimated at 7.5 ug per ml culture supernatant. By use of the double antibody sandwich ELISA, the results of this preliminary investigation indicate that at this trypsin to alpha toxin ratio, digestion of the toxin was initiated at around 5 minutes and was complete at around 35 minutes (figure 2. 1). Therefore, it seemed possible that sometime between 5 and 35 minutes, a fragment containing a single antigenic determinant might have been generated. Screening of each sample with the competitive enzyme– linked immunoassay confirmed that the 20, 25, 30, and 35 minute samples contain such fragments (figure 2. 2). In these sample wells, the absorbance values were close to zero. This indicated that the available sites on the adsorbed antitoxin were occupied by fragments of toxin that were incapable of binding the second enzyme– labelled antibody, which resulted in no substrate hydrolysis in these wells. These results indicate that it was possible to obtain immunologically reactive fragments with trypsin and to detect these fragments with a soluble immunoassay. Further isolation and identification of these fragments was performed with an SDS– PAGE/ immuno electrotransfer blot system.

Digestion of alpha toxin with trypsin (1 % by weight of protein)

Purified alpha toxin was digested with trypsin (1 % by weight of protein) for a period of 2 hours at 37 C. The reaction was stopped by the addition of soybean trypsin inhibitor (2 % by weight of protein) and the fragments lyophilized and reconstituted in water. The fragments were then separated and examined with an SDS- PAGE immuno electrotransfer blot system. In this assay the immunologically reactive fragments were identified according to their reaction with whole antitoxin as well as purified antibinding and indirect hemagglutinating antibodies. The results indicate that although two immunologically reactive fragments were obtained, the bulk of the toxin remained undigested (figure 2. 3). Both of the fragments reacted with whole antitoxin as well as with antibinding antibodies. The approximate molecular weights of these fragments were 31, 000 and 20, 000 Daltons. Similar results were obtained when the digestion time was increased to 6 hours.

Digestion of alpha toxin with trypsin (5 % by weight of protein)

Due to the inability of trypsin to fully digest the toxin when used at a concentration of 1 % by weight of the protein, it was decided to increase the trypsin concentration to 5 % by weight of the protein. Purified alpha toxin was digested with trypsin (5 % by weight of protein) with samples removed at 1, 2.5, and 6 hours. The reaction was stopped at each time by the addition of soybean trypsin inhibitor (10 % by weight of protein). The fragments obtained were lyophilized and reconstituted in water. They were then separated and examined in an SDS- PAGE immuno electrotransfer blot system. In this assay, the immunologically reactive fragments were identified according to their reaction with

whole antitoxin, and purified preparations of antibinding and indirect hemagglutinating antibodies. The results indicate that at one hour the toxin was digested to yield 6 small fragments (figure 2. 4). The approximate molecular weights of these fragments ranged from approximately 11, 000 to 16, 000 Daltons. Five of the six fragments reacted with antibinding antibodies and at least two reacted with indirect hemagglutinating antibodies. In addition three larger fragments were also produced (data not shown). Two of these fragments appeared as closely spaced lines at around 31, 000 to 32, 000 Daltons. The third fragment had a molecular weight of approximately 20, 000 Daltons. All three of these fragments were recognized by both antibinding and indirect hemagglutinating antibodies. At 2. 5 hours, all of the toxin was digested to yield two large fragments of approximately 31, 000 and 32, 000 Daltons and a smaller fragment of approximately 20 kiloDaltons (figure 2. 5). All three of these fragments were recognized by both antibinding and indirect hemagglutinating antibodies. At 6 hours, the 20, 000 Dalton fragment was still present. However, the larger fragments were slightly degraded to yield a 30, 0000 Dalton fragment (figure 2. 6). Both of these fragments were recognized by antibinding and indirect hemagglutinating antibodies.

Digestion of alpha toxin with cyanogen bromide

Alpha toxin was digested with cyanogen bromide (100 fold molar excess over methionine residues) for 48 hours. The fragments obtained were then diluted 15–fold with water, shell frozen and lyophilized. After reconstitution in water, the fragments were isolated and examined with an SDS– PAGE immuno electrotransfer blot system. As shown in figure 2. 7A, 5 fragments that were recognized by whole antitoxin were obtained. The approximate molecular weights

of these fragments were 9, 000; 10, 000; 10, 500; 11, 000 and 12, 000 Daltons. The 9, 000; 10, 000 and 12, 000 Dalton fragments were recognized by antibinding antibodies (figure 2. 7B) and the 9, 000; 10, 000; 10, 500, and 11, 000 Dalton fragments were recognized by indirect hemagglutinating antibodies (figure 2. 7C).

Identification of a CNBr generated binding fragment of alpha toxin

Further examination of the cyanogen bromide generated fragments in the kinetic hemolysis assay, revealed that none of the fragments contained any hemolytic activity as compared to native toxin (figure 2. 8). However, preincubation of rabbit erythrocytes with the fragments protected the erythrocytes from lysis by subsequently added intact alpha toxin (figure 2. 9). This indicated that one of the cyanogen bromide fragments of alpha toxin contained the site responsible for binding to the erythrocyte receptor. When the fragment preparation was incubated with rabbit erythrocyte membranes for 10 minutes and then centrifuged, the fragments that remained in the supernatant, no longer protected rabbit erythrocytes from toxin– mediated lysis (figure 2. 10). This indicated that the binding fragment was adsorbed out by the erythrocyte membranes. SDS–PAGE immuno electrotransfer blot examination of the fragment populations before and after incubation with rabbit erythrocyte membranes, revealed that the 9, 000 Dalton fragment was removed (figure 2. 11). Thus it seems that this 9, 000 Dalton CNBr generated fragment of alpha toxin contains the site responsible for binding to the rabbit erythrocyte receptor.

Figure 2. 1

Double antibody sandwich ELISA for detection of trypsin (trypsin 1 % by weight of toxin) digested alpha toxin.

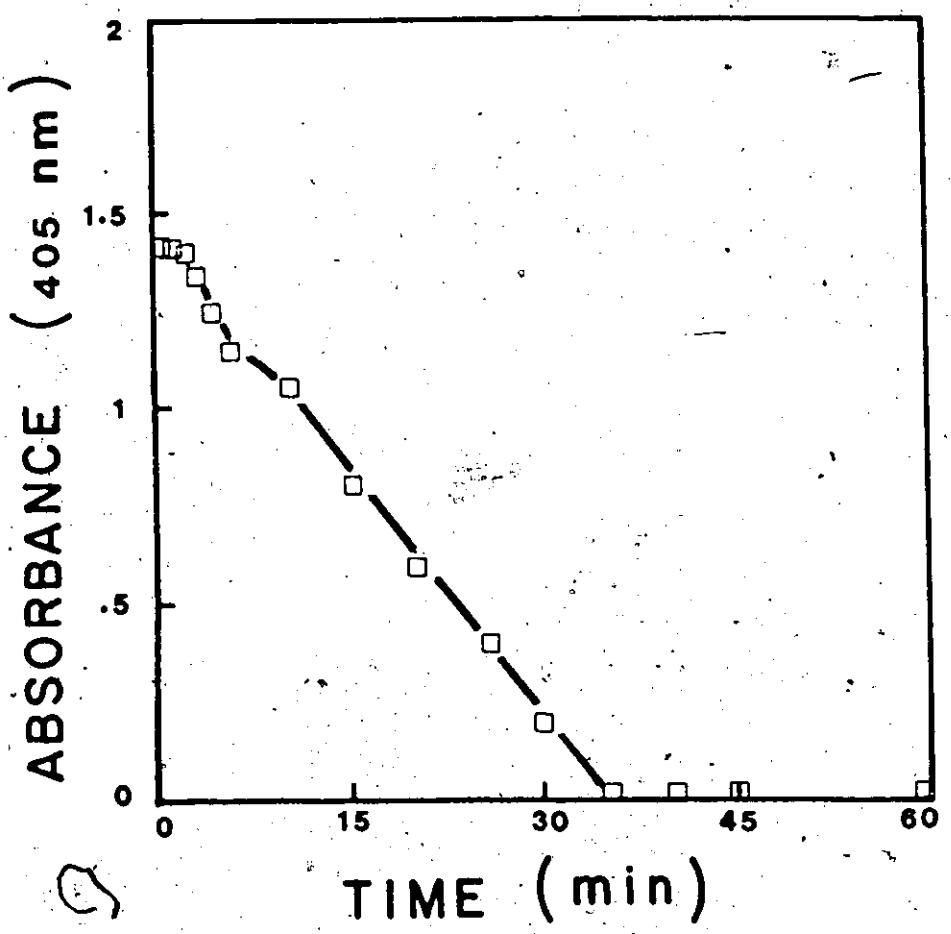

ABSORBANCE (405 nm)
2
1.5
1
.5
0
0
15
30
45
60
TIME (min)

Figure 2. 2

Competitive enzyme- linked immunoassay (CELIA) for the detection of immunologically reactive fragments of alpha toxin that were obtained by digestion with trypsin (trypsin 1 % by weight of toxin). The 20, 25, 30 and 35 minute samples contain such fragments as evidenced by the near zero absorbance values in these wells.

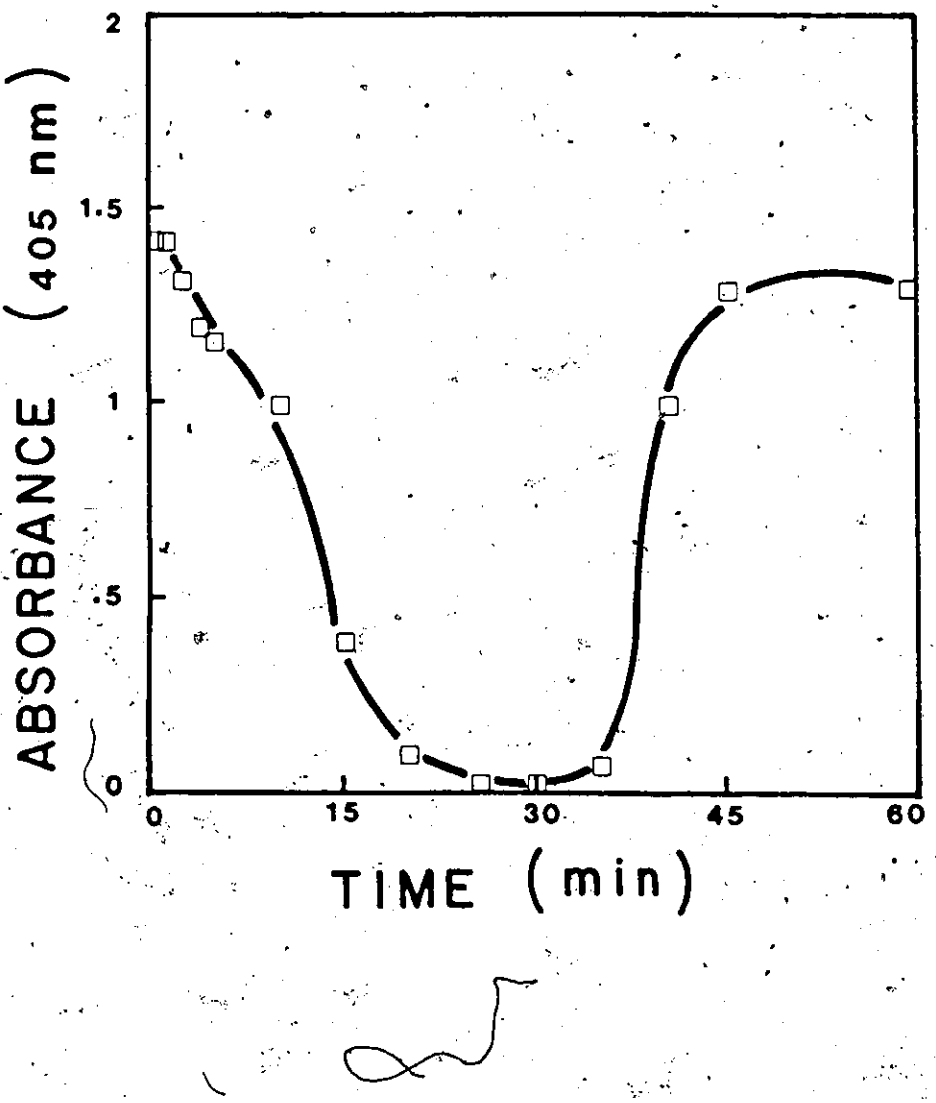
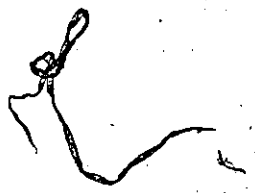

ABSORBANCE (405 nm)
2
1.5
1
.5
0
0
15
30
45
60
TIME (min)

Figure 2. 3



Demsitometric scans of SDS- PAGE/ immuno electrotransfer blots of trypsin (trypsin 1 % by weight of toxin) digested alpha toxin. Molecular weights are expressed in kiloDaltons. The x- axes represent distance migrated from the top of the gel (cm). The y- axes represent relative absorbance (600 nm). Panels A, B, and C show fragments that were detected with whole antitoxin, purified antibinding antibodies, and purified indirect hemagglutinating antibodies respectively.

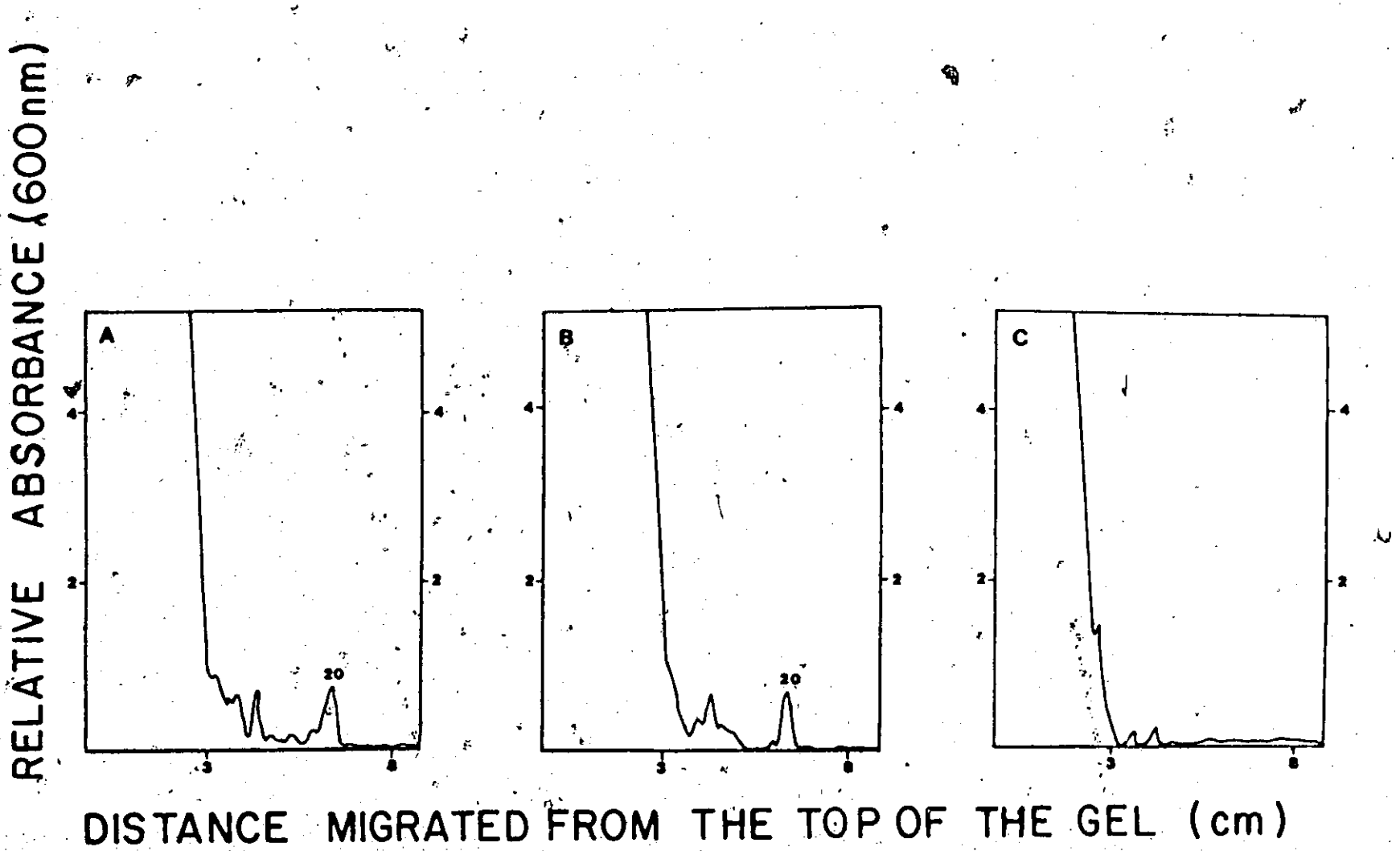

RELATIVE ABSORBANCE (600nm)
A
B
C
4
2
20
3
8
DISTANCE MIGRATED FROM THE TOP OF THE GEL (cm)

Figure 2. 4

Densitometric scans of SDS/ PAGE immuno electrotransfer blots of alpha toxin that was digested for 1 hour with trypsin (trypsin 5 % by weight of toxin). Molecular weights are expressed in kiloDaltons. The x– axes represent distance migrated from the top of the gel (cm). The y– axes represent relative absorbance (600 nm). Panels A, B, and C show fragments that were detected with whole antitoxin, purified antibinding antibodies and purified indirect hemagglutinating antibodies respectively.

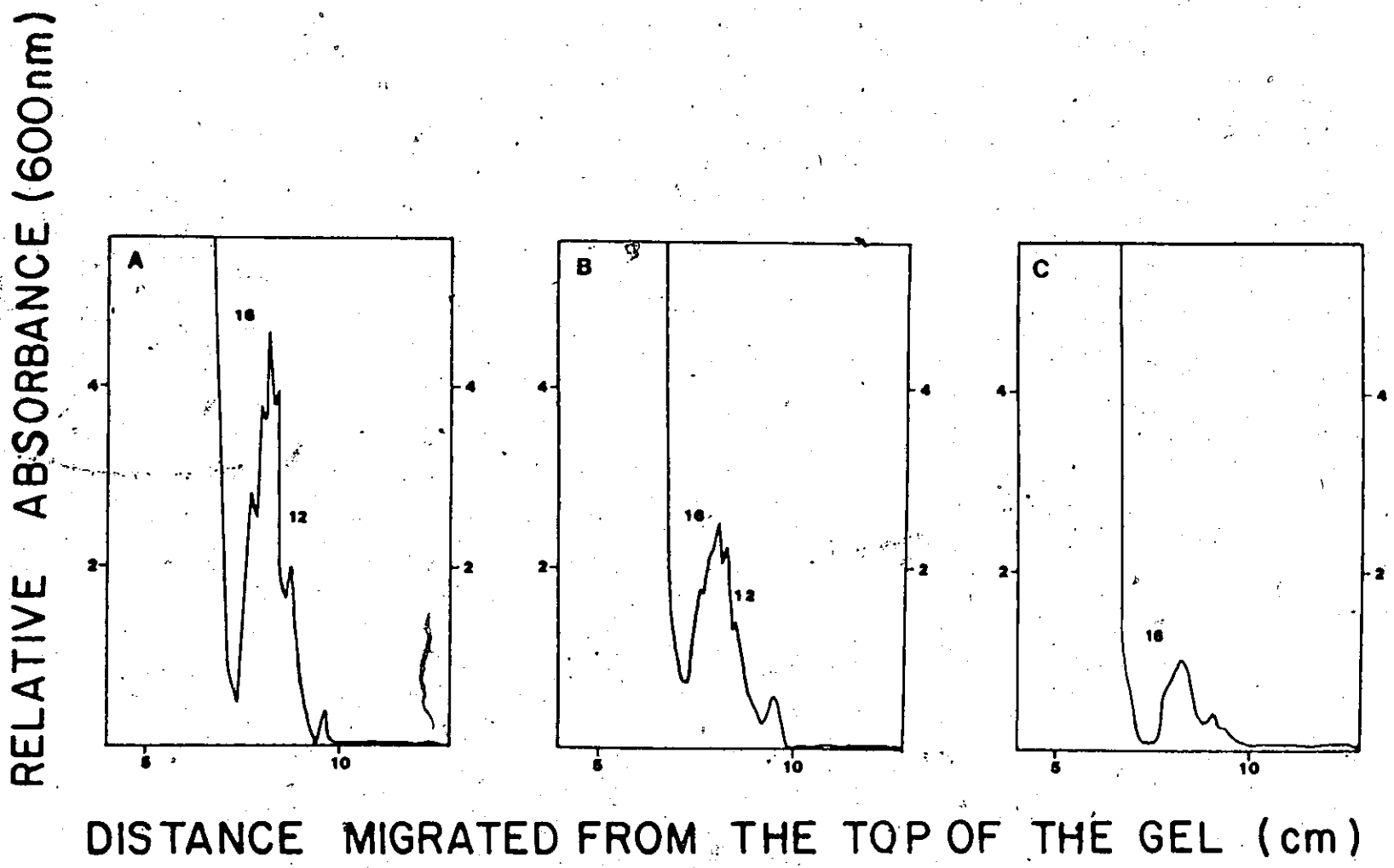

RELATIVE ABSORBANCE (600nm)
A
B
C
16
12
16
12
16
4
2
5
10
DISTANCE MIGRATED FROM THE TOP OF THE GEL (cm)

Figure 2. 5

Densitometric scans of SDS/ PAGE immuno electrotransfer blots of alpha toxin that was digested for 2. 5 hours with trypsin (trypsin 5 % by weight of toxin). Molecular weights are expressed in kiloDaltons. The x– axes represent distance migrated from the top of the gel (cm). The y– axes represent relative absorbance (400 nm). Panels A, B, and C show fragments that were detected with whole antitoxin, purified antibinding antibodies and purified indirect hemagglutinating antibodies respectively.

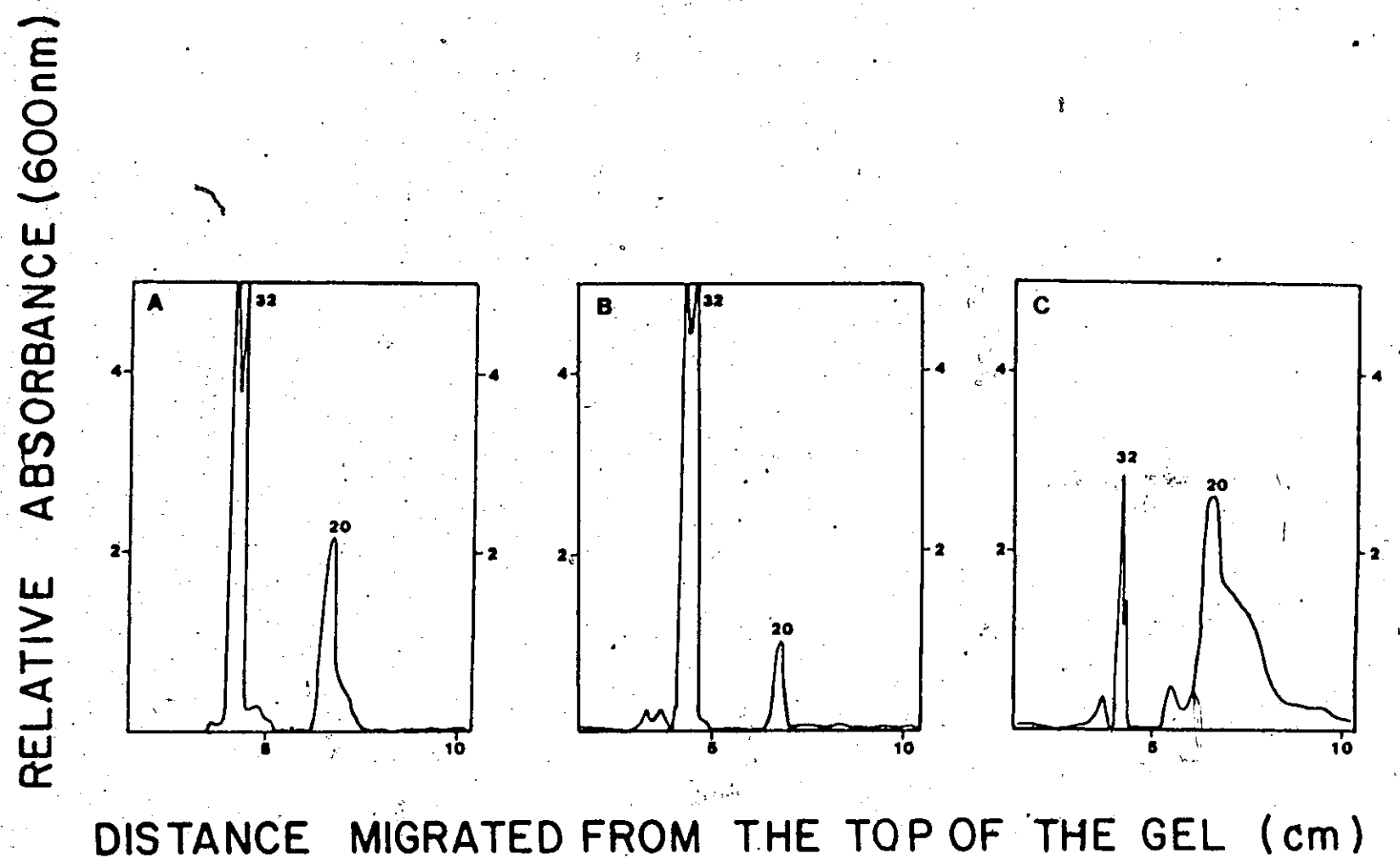

RELATIVE ABSORBANCE (600nm)
A
B
C
32
20
4
2
5
10
DISTANCE MIGRATED FROM THE TOP OF THE GEL (cm)

Figure 2. 6

Densitometric scans of SDS/ PAGE immuno electrotransfer blots of alpha toxin that was digested for 6 hours with trypsin (trypsin 5 % by weight of toxin). Molecular weights are expressed in kiloDaltons. The x– axes represent distance migrated from the top of the gel (cm). The y– axes represent relative absorbance (600 nm). Panels A, B, and C show fragments that were detected with whole antitoxin, purified antibinding antibodies, and purified indirect hemagglutinating antibodies respectively.

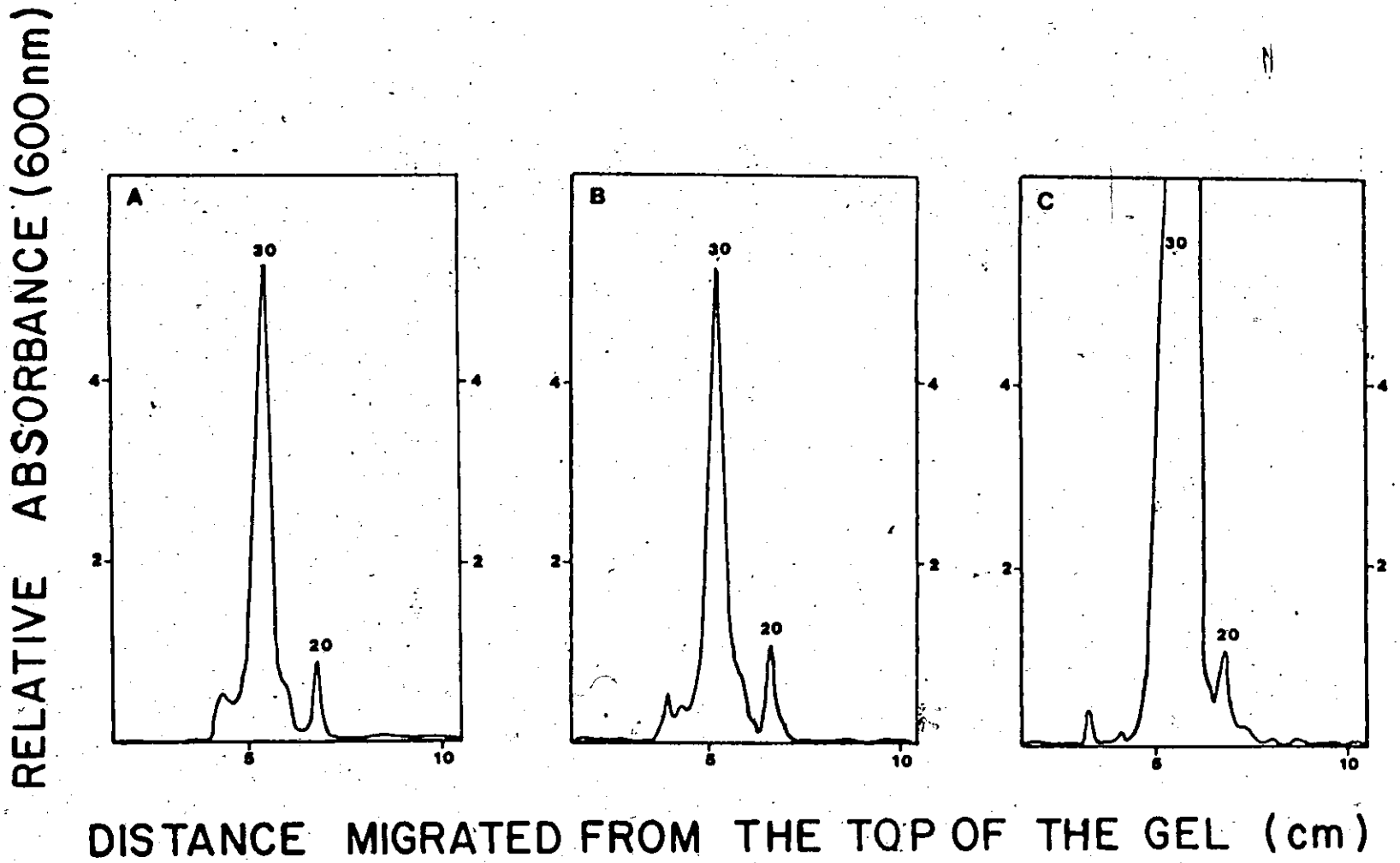

RELATIVE ABSORBANCE (600nm)
A
B
C
30
20
2
4
5
10
DISTANCE MIGRATED FROM THE TOP OF THE GEL (cm)

Figure 2.7

Densitometric scans of SDS/ PAGE immuno electrotransfer blots of alpha toxin that was digested for 48 hours with cyanogen bromide (100 fold molar excess over methionine residues). Molecular weights are expressed in kiloDaltons. The x– axes represent distance migrated from the top of the gel (cm). The y– axes represent relative absorbance (600 nm). Panels A, B, and C show fragments that were detected with whole antitoxin, purified antibinding antibodies, and purified indirect hemagglutinating antibodies respectively.

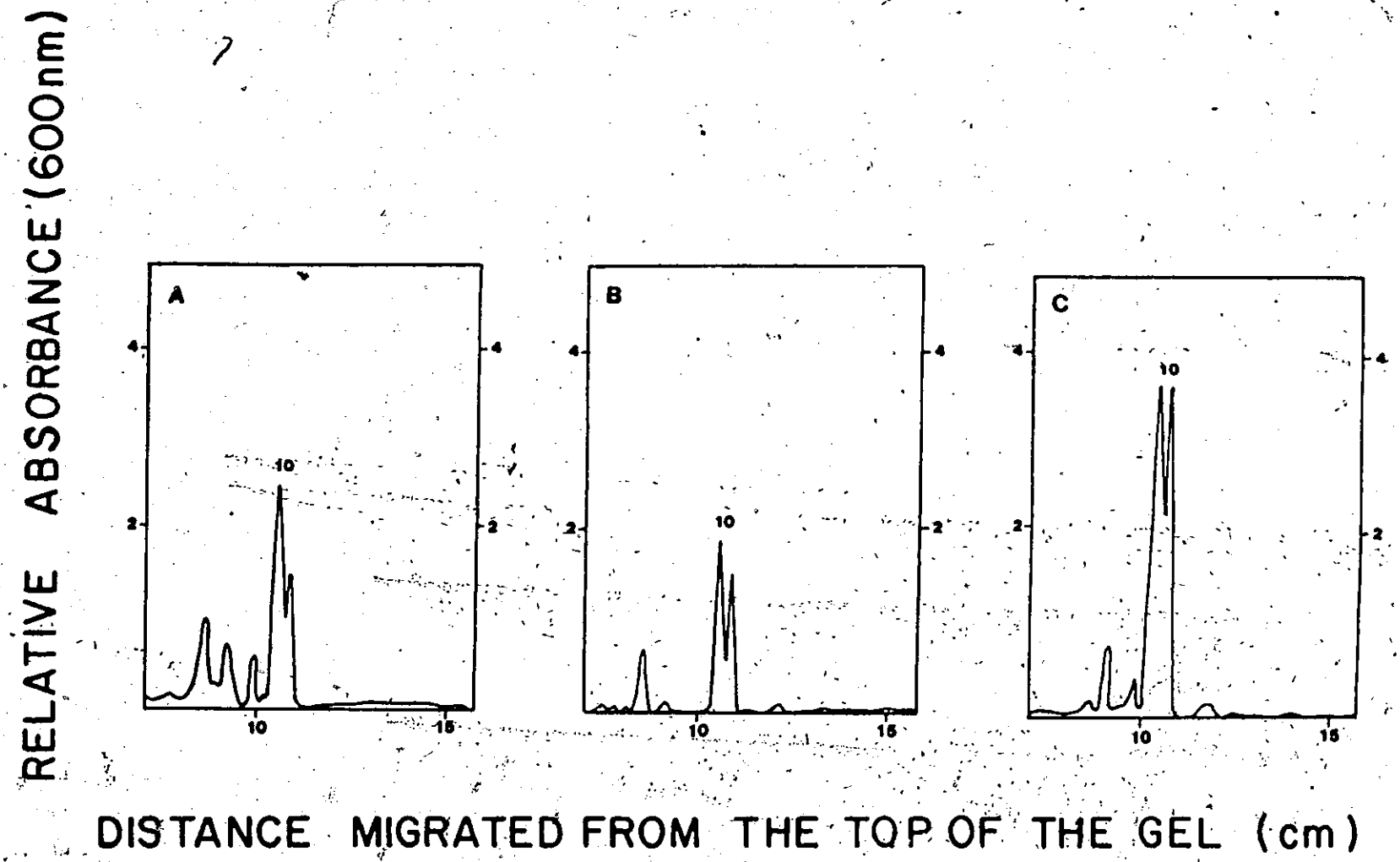

RELATIVE ABSORBANCE (600nm)
A
B
C
4
2
10
15
DISTANCE MIGRATED FROM THE TOP OF THE GEL (cm)

Figure 2. 8

Lysis of rabbit erythrocytes by alpha toxin measured turbidometrically (650 nm) over time. Line A represents the control, in which intact alpha toxin was added to the erythrocytes. Line B shows the test, in which the cyanogen bromide fragments of alpha toxin were added to the erythrocytes.

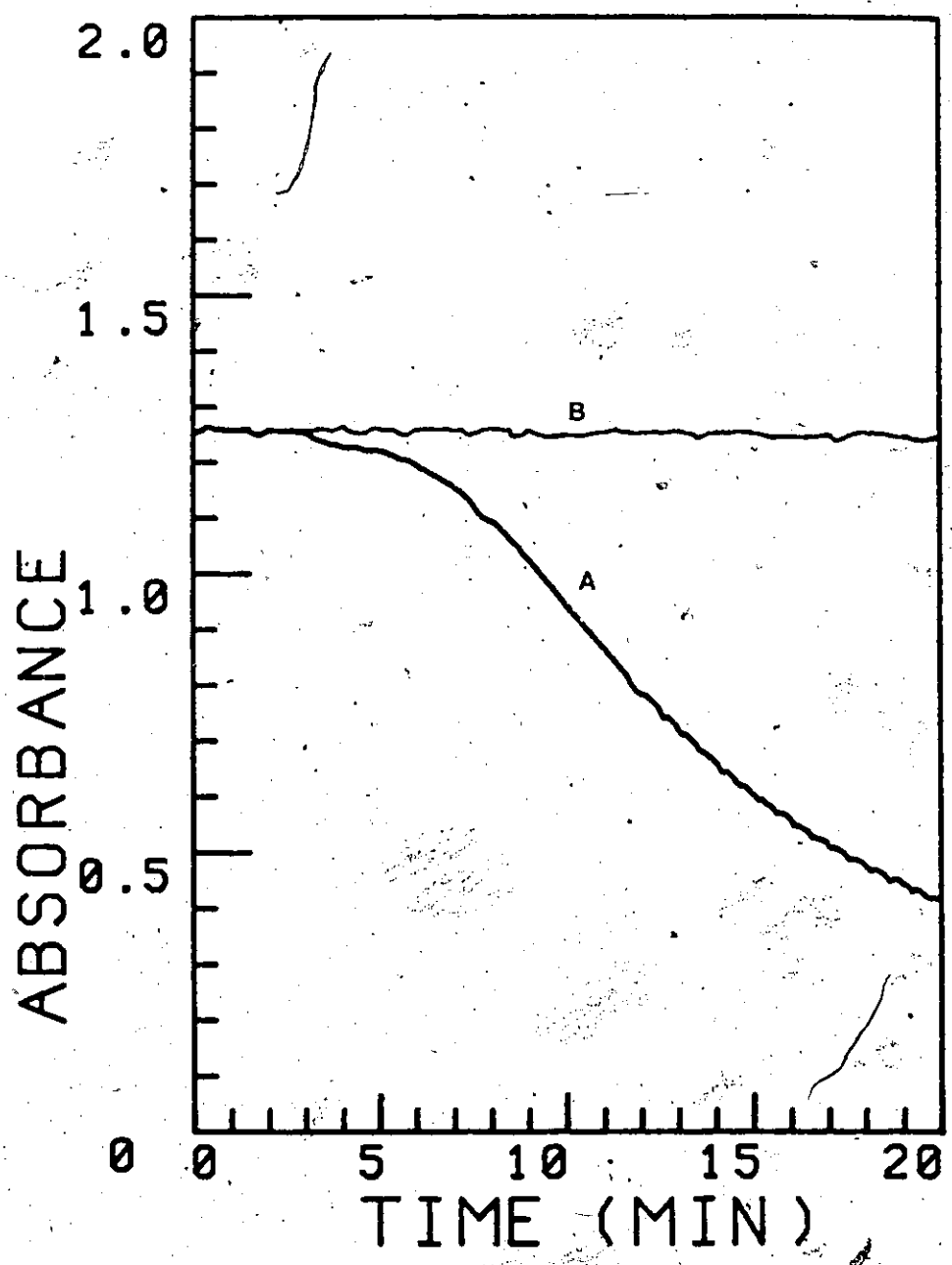

2.0
1.5
1.0
0.5
0
ABSORBANCE
B
A
0
5
10
15
20
TIME (MIN)

Figure 2. 9

Lysis of rabbit erythrocytes by alpha toxin measured turbidometrically (650 nm) over time. Line A represents the control, in which intact alpha toxin was added to the erythrocytes. Line B shows the test, in which the erythrocytes were preincubated with the CNBr generated alpha toxin fragments for 10 minutes, and then challenged with the same amount of intact toxin that was used in A.

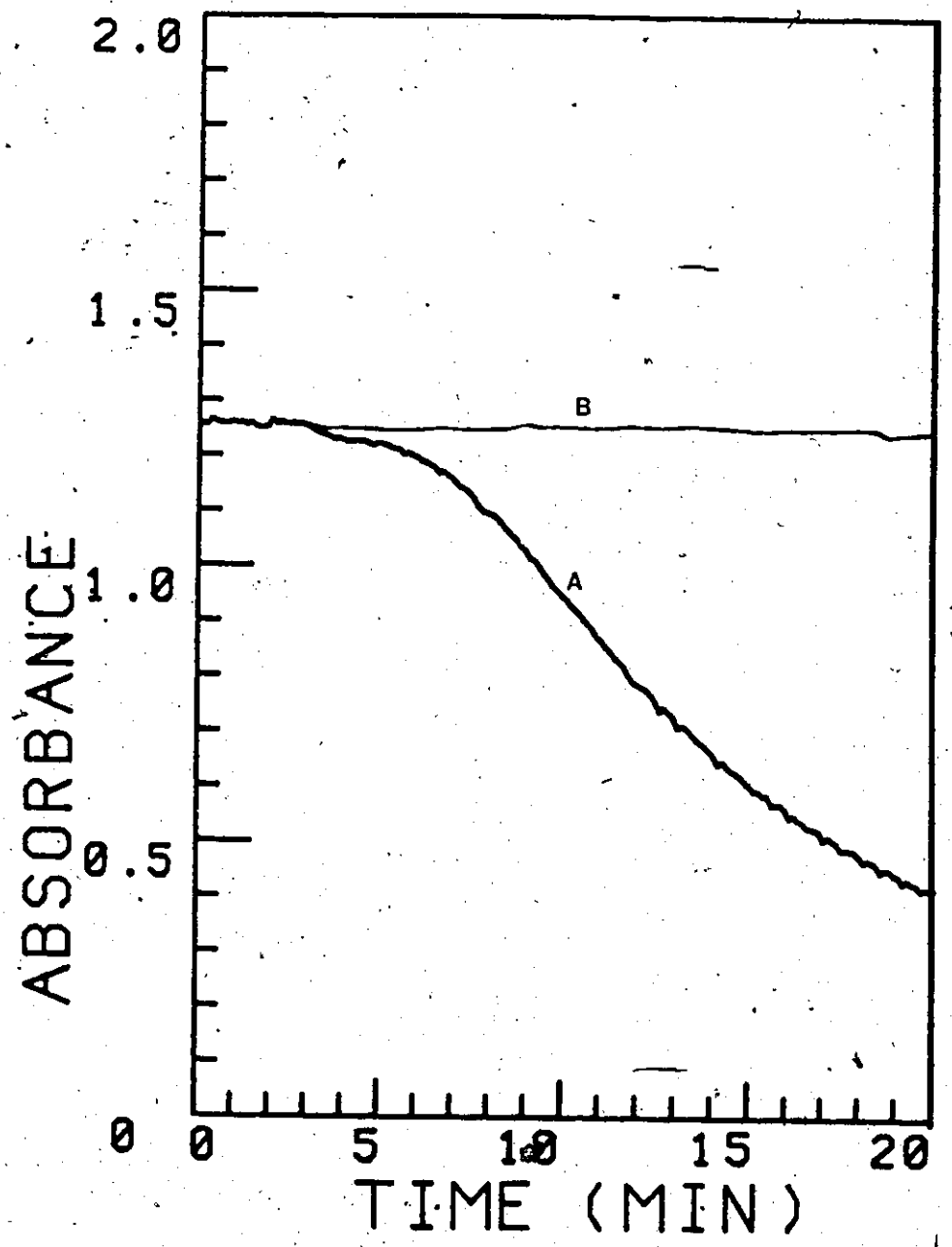

2.0
1.5
1.0
0.5
0
ABSORBANCE
B
A
0
5
10
15
20
TIME (MIN)

Figure 2. 10

Lysis of rabbit erythrocytes by alpha toxin measured turbidometrically (650 nm) over time. The erythrocytes used in this assay were preincubated for 10 minutes with rabbit erythrocyte membrane– adsorbed cyanogen bromide generated fragments of alpha toxin. Intact alpha toxin was then added, and the amount of lysis measured.

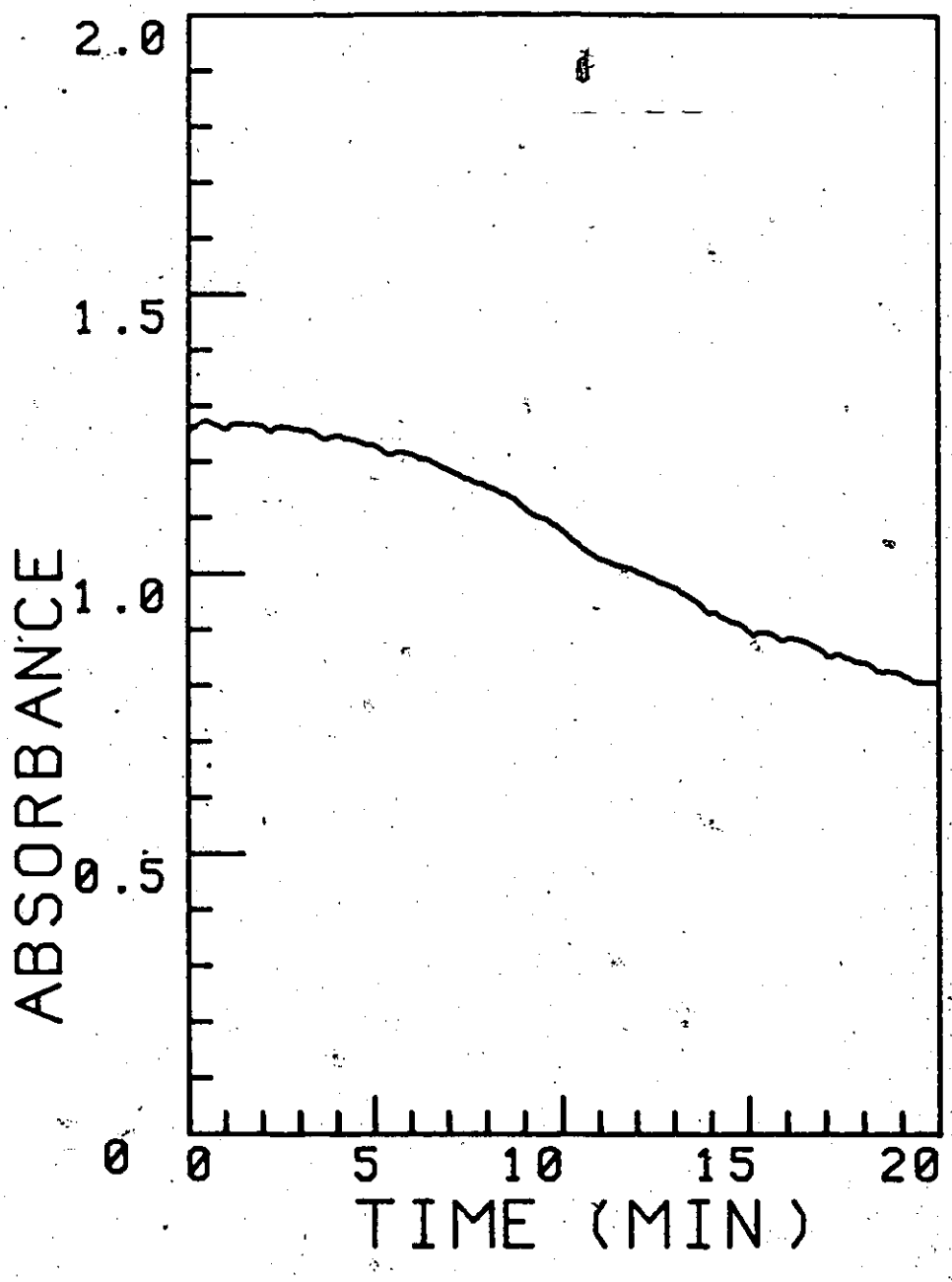
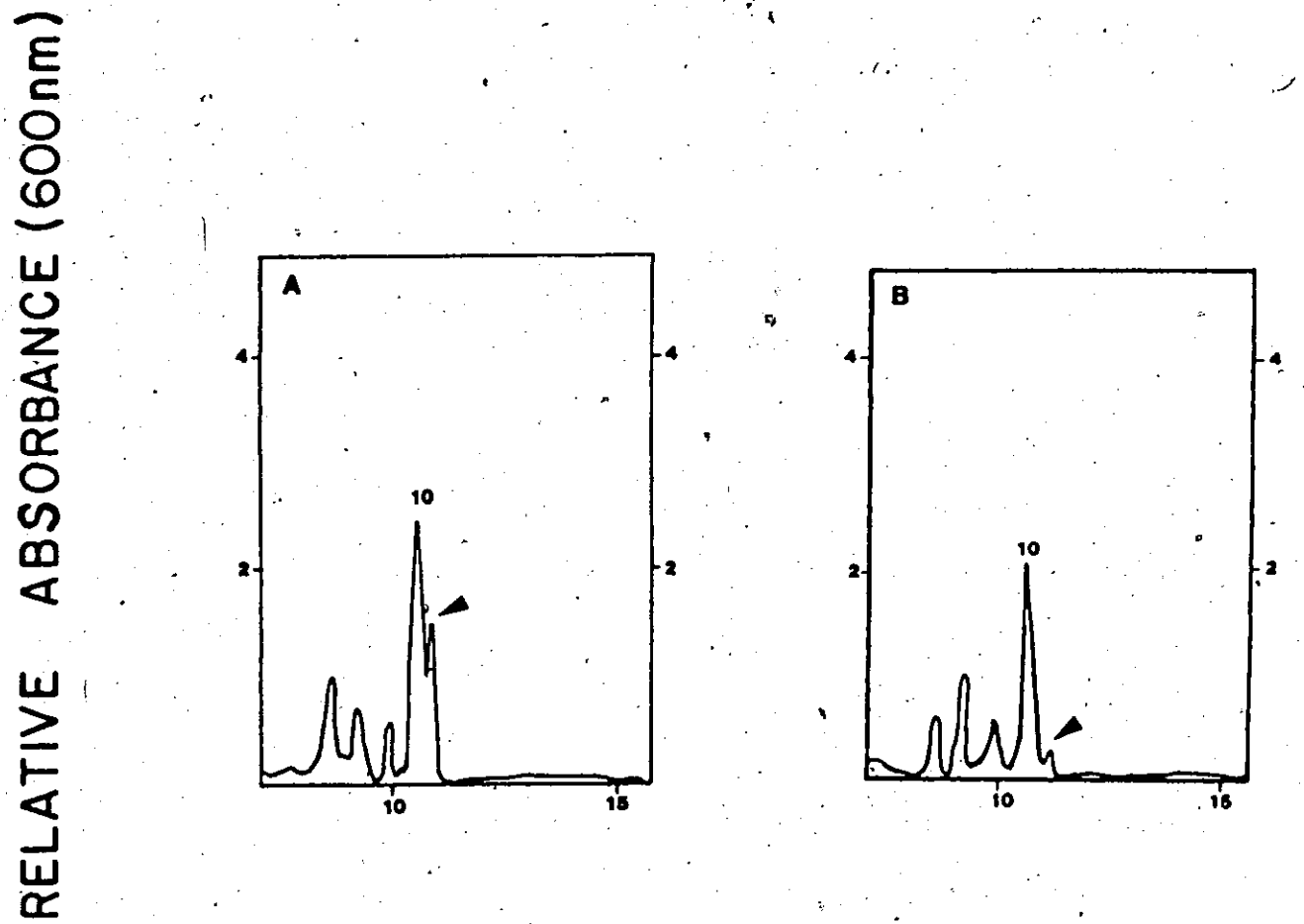

2.0
1.5
1.0
0.5
0
ABSORBANCE
0
5
10
15
20
TIME (MIN)

Figure 2. 11.

Densitometric scans of SDS/ PAGE immuno electrotransfer blots of alpha toxin that was digested with cyanogen bromide (100 fold molar excess over methionine residues). The molecular weights are expressed in kiloDaltons. The x– axes represent the distance migrated from the top of the gel (cm). The y– axes represent the relative absorbance (600 nm). Panel A shows the entire population of fragments as detected by whole antitoxin. Panel B is the same as panel A, except that the fragments were adsorbed for 10 minutes with a rabbit erythrocyte membrane preparation prior to electrophoresis. The arrow shows a 9 kiloDalton fragment that was lost due to the rabbit erythrocyte membrane– adsorption step.


RELATIVE ABSORBANCE (600nm)
A
B
4
2
10
15
DISTANCE MIGRATED FROM THE TOP OF THE GEL (cm)

Discussion

In this study, alpha toxin was subjected to digestion with trypsin, alpha chymotrypsin and cyanogen bromide in order to obtain fragments which might be more amenable to structural analysis. In order to obtain fragments of a fairly large size, cyanogen bromide was chosen as a degradation agent since only approximately seven methionine residues exist in alpha toxin. The proteases were chosen to generate smaller sized fragments since alpha toxin possesses approximately 38 potential tryptic cleavage sites and approximately 54 potential chymotryptic cleavage sites.

Digestion of alpha toxin with alpha chymotrypsin under the same conditions as those used for trypsin did not yield any immunologically reactive fragments (data not shown). This was probably due to cleavage at a large number of sites in the molecule, which produced very small fragments. Dalen (1976b) reported that his alpha toxin (MW 39, 000) was degraded by chymotrypsin to yield a 27, 500 Dalton fragment that was resistant to further chymotrypsin digestion. Watanabe and Kato (1978) on the other hand, reported that their alpha toxin (MW 36, 000) was resistant to chymotrypsin. The differences in our results are probably due to the fact that, unlike both of these groups, our alpha toxin (MW 34, 000) was extensively denatured prior to addition of the enzyme.

Digestion of alpha toxin with 1 % trypsin for 2 hours yielded two fragments with approximate molecular weights of 31, 000 and 20, 000 Daltons. Both of these fragments were recognized by antibinding antibody. Increasing the concentration of trypsin to 5 % initially yielded six small fragments, the molecular weights of which ranged from 12, 000 to 16, 000 Daltons. Five of these fragments were recognized

by antibinding antibodies, and at least two by indirect hemagglutinating antibodies. In addition, three larger fragments were also found (data not shown). Two of these fragments appeared as closely spaced lines at around 31, 000 to 32, 000 Daltons, which probably indicate a sequential loss of peptides from the toxin. A similar sequential loss of peptides was noted by Dalen (1976b) when he examined the degradation of alpha toxin by natural proteases. The third large fragment had a molecular weight of approximately 20, 000 Daltons. All three of these large fragments were recognized by both antibinding and indirect hemagglutinating antibodies. Further digestion of the toxin with 5 % trypsin resulted in the six small fragments being lost. However, the three large fragments persisted. Finally, digestion of the toxin with 5 % trypsin for six hours, resulted in a 30, 000 Dalton fragment which probably resulted from further sequential degradation of the 31, 000 and 32, 000 dalton fragments, and, the 20, 000 Dalton fragment. Both of these fragments were recognized by antibinding and indirect hemagglutinating antibodies.

The common and most striking feature of these results is the 20, 000 dalton fragment. This fragment appeared early in the digestion course and seems to be resistant to further digestion with trypsin. The biological properties of this fragment are as yet unknown. Watanabe and Kato (1978) reported a 17, 000 Dalton tryptic fragment of alpha toxin that is resistant to trypsin digestion. Their fragment was lethal but non- dermonecrotic and non- hemolytic. Blomqvist and Thelestam (1986a) reported a fragment of apparent molecular weight 18, 500 Daltons that appeared naturally during their purification of alpha toxin. This fragment was non- lethal but was hemolytic and was capable of binding to Y1 cells and the mouse target organ. We have also observed a similar sized contaminating fragment in SDS/ PAGE immuno electrotransfer blots of purified alpha toxin (data not shown). However, since we have not yet investigated the

biological properties of this fragment, comparisons cannot be made with the fragment of Blomvqist and Thelestam (1986a).

Cyanogen bromide fragmentation of alpha toxin produced five immunologically reactive fragments with approximate molecular weights of 9, 000; 10, 000; 10, 500; 11, 000 and 12, 000 daltons. The 9, 000; 10, 000 and 12, 000 dalton fragments reacted with antibinding antibodies. The 9, 000; 10, 000; 10, 500, and 11, 000 dalton fragments reacted with indirect hemagglutinating antibodies. The fact that the sum of the apparent molecular weights of the peptides is greater than the molecular weight of native intact alpha toxin may be explained in several ways. Firstly, there may have been overlapping fragments as a result of incomplete digestion. However, this possibility has been eliminated due to prolonged digestion of the toxin with cyanogen bromide. The same number of fragments were detected after 30 and 48 hour digestions. Secondly, the molecular weights expressed in this report are only approximate due to differential shrinkage of the nitrocellulose when stained for total protein (standards) and when immunostained (peptides). Finally, in SDS PAGE, the linear correlation between the log of the molecular weight and distance of migration in the gel, that is found with larger proteins may not be true for smaller peptides. The deviations from linearity may be due to a complex interplay of intrinsic charge, differential SDS binding and peptide shape (Swank and Munkries, 1971). Six and Harshman (1972b) reported a total of seven fragments that were obtained by cyanogen bromide digestion of alpha toxin. These fragments were numbered I to VII in order of their elution from a Sephadex G– 50 column. These fragments were further examined by a soluble ELISA and a SDS PAGE/ immuno electrotransfer blot system for their reactivity with rabbit polyclonal antisera and a monoclonal antibody (Harshman *et al.*, 1986). It was found that fragments IV, V and VII reacted with their rabbit antiserum and fragment V

reacted with their monoclonal antibody. However, since the relative molecular weights of Harshman's (1986) fragments are not available, and it is not clear whether the numbering scheme used, is the same in the gel filtration and the SDS PAGE, it is difficult to compare his fragments and ours. The greater number of fragments that is recognized by our polyclonal antisera may be explained in two ways. Firstly, different antisera may recognize different antigenic determinants (Atassi, 1975; Atassi and Saplin, 1968). Secondly, our polyclonal antisera was prepared by injecting toxin that was denatured by boiling prior to injection. This may have resulted in the exposure of a greater number of determinants and the corresponding production of a greater variety of antitoxins in our preparation.

The number of immunologically reactive fragments of alpha toxin that were obtained by both tryptic and cyanogen bromide digestion, indicate that the number of antigenic determinants in this molecule is greater than that previously calculated. Lo (1984, PhD Dissertation) reported that there was one indirect hemagglutinating and two antibinding determinants in alpha toxin. The difference in results may be attributed to any of four possible reasons. Firstly, Lo (1984) only measured the number of epitopes on alpha toxin. Since the toxin used in this study was fragmented, cryptotopes were also accounted for. Secondly, different antisera may recognize different antigenic determinants (Atassi, 1975; Atassi and Saplin, 1968). Thirdly, the possibility exists that some determinants may be so close together that an antibody bound to one would sterically hinder access to adjacent determinants (Atassi, 1967; 1975). This constraint was lifted when the molecule was fragmented. Finally, a recent study on the structure and assembly mechanism of alpha toxin, suggests that the toxin, upon binding to its membrane receptor, undergoes a change in tertiary structure (Tobkes *et al.*, 1985). In a model proposed by these authors, the toxin turns partly inside out before forming

hexamers in which the hydrophilic residues that were formerly on the surface of the monomer line the channel or take part in subunit– subunit or domain– domain interactions. Blomqvist and Thelestam (1986b) showed that after binding to Y1 cell membrane, the toxin undergoes a conformational change at the cell surface before any membrane damage occurs. Since the indirect hemagglutinating antibodies can only be isolated from toxoid already bound to erythrocytes, it is possible that the IHA determinants are only exposed after the toxin is bound to its membrane receptor and undergoes a conformational change. These determinants would not be available on native toxin in solution. This would account for Lo's (1984, PhD Dissertation) data and also account for the increase in the apparent number of IHA determinants seen in this study.

Alpha toxin mediated lysis of red blood cells is currently believed to involve the following sequence of steps: (1) binding of the toxin monomers to specific receptors on the cell surface, (2) formation of a hexamer complex which penetrate the lipid bilayer to form transmembrane channels, (3) leakage of small ions through the channels, and (4) colloid osmotic lysis of the cell (Bhakdi *et al.*, 1984; Blomqvist and Thelestam, 1986a; Cassidy and Harshman, 1976b; Cassidy and Harshman, 1979; Freer and Arbuthnott, 1983; Harshman, 1979; Harshman *et al.*, 1986). With the recent evidence of Blomqvist and Thelestam (1986b) and Tobkes *et al.*, (1985) another step in the sequence of events leading to hemolysis may be added. This step which involves a conformational change in the toxin most likely occurs after binding of the toxin and before hexamer formation. Since the indirect hemagglutinating antibodies prevent lysis of the cell after the toxin has already been bound, it seems feasible that these antibodies act by either preventing the conformational change from occuring or, by sterically preventing the protein–protein interactions that are necessary for hexamer formation.

In this study a 9, 000 Dalton cyanogen bromide generated fragment of alpha toxin that bound to the rabbit erythrocyte receptor was isolated. As determined by the kinetic hemolysis assay this fragment was non- hemolytic. However, the other biological properties of this fragment were not investigated. Recently, Blomqvist and Thelestam (1986a) proposed an hypothetical map of the biologically active regions of alpha toxin. This map was based on their investigation of a 18, 500 dalton fragment of the toxin and previous fragmentation analyses performed by Kato and Watanabe (1980) and Watanabe and Kato (1978). In this model, alpha toxin and its fragments are depicted as straight polypeptides of lengths corresponding to their molecular weights. The binding region is thought to lie in the middle of the molecule. Furthermore, Blomqvist and Thelestam (1986a) suggest that the binding region may correspond to the hinge region that Tobkes *et al*, (1985) postulated to be near the midpoint of the alpha toxin polypeptide chain. Our 9, 000 dalton binding fragment may represent a portion of Blomqvist and Thelestam's (1986a) fragment which has lost its hemolytic properties. Whether or not our fragment is dermonecrotic awaits further purification. Thus our fragment fits in with Blomqvist and Thelestam's (1986a) model and supports the notion of a discrete binding region on the alpha toxin polypeptide chain.

CHAPTER THREE

Production and characterization of monoclonal antibodies to staphylococcal alpha toxin

Introduction

Alpha toxin is an extracellular protein that is produced by most strains of *Staphylococcus aureus* (Baird- Parker, 1972; 1974; Kloos and Schleifer, 1986). This protein is considered to be the most toxic product of these organisms (Arbuthnott, 1970). This toxin is hemolytic, dermonecrotic, cytotoxic for a wide variety of cells, and lethal for most laboratory animals (Cassidy *et al.*, 1974; Freer and Arbuthnott, 1983; Harshman, 1979; Jeljaszewicz, 1972; Rogolsky, 1979; Thelestam, 1983). Alpha toxin consists of a single polypeptide chain, which is devoid of any disulfide bonds or sulfhydral groups (Arbuthnott, 1970; Bernheimer *et al.*, 1963; Lominski *et al.*, 1963; Six *et al.*, 1973a, b). The molecular weight of this protein is currently believed to be in the range of 28, 000 (Six and Harshman, 1973b) to 34, 000 (Freer and Arbuthnott, 1983; Thelestam, 1983; Bhakdi and Tranum- Jensen, 1986) Daltons. The amino acid composition of this protein has been analyzed by several groups, and the results are in reasonable agreement (Bernheimer and Schwartz, 1963; Coulter, 1966; Fackrell and Wiseman, 1973a; Kato and Watanabe, 1980; Six and Harshman 1973a; Watanabe and Kato, 1978; Wiseman and Caird, 1970).

It is currently believed that the varoius biological activities that are exhibited by alpha toxin are a result of toxin- membrane interactions. These interactions has been extensively studied in hemolytic systems primarily using rabbit erythrocytes (Barei and Fackrell, 1979; Cassidy and Harshman, 1976a, b; Cooper *et al.*, 1964a; Klainer *et al.*, 1964; Lo and Fackrell, 1980; Lominski and Arbuthnott,

1962; Madoff *et al*, 1964; Maharaj and Fackrell, 1980; Marucci, 1963a, b; Mungalo and Renaud, 1959). Maharaj and Fackrell (1980) have shown that the erythrocyte membrane receptor of alpha toxin is the transmembrane protein, band three. More recent studies have further narrowed the location of the receptor to be the carbohydrate moiety of band three (Simpson, 1986, MSc Thesis). Thelestam (1983b) has also suggested that a receptor for alpha toxin exists on cultured mouse adrenocortical (Y1) cells.

Studies have suggested that separate regions on the alpha toxin molecule are responsible for receptor binding and membrane damage in rabbit erythrocytes (Barei and Fackrell, 1979; Cassidy and Harshman, 1976; Lo and Fackrell, 1980) and in Y1 cells Thelestam and Blomqvist (1984). Fragmentation analysis have also suggested that different regions on the molecule are responsible for hemolysis and lethality (Blomqvist and Thelestam, 1986a; Kato and Watanabe, 1980; Watanabe and Kato, 1978). Blomqvist and Thelestam (1986a) have postulated that all four of the biological effects of alpha toxin may be initiated by binding of the toxin *via* a single binding region. Furthermore, these authors have suggested that the binding region may be identical with a putative hinge region located near the centre of the molecule, that was reported by Tobkes *et al*, (1985).

So far, there has been only one report of a structure- function study of alpha toxin in which antibodies have been utilized (Harshman *et al*, 1986). These authors used a monoclonal antibody that bound to an epitope in the carboxyl terminus of the toxin. Since the antibody did not impede binding of the toxin to the receptor, and did not inhibit hemolysis or hexamer formation, it was concluded that these functions were not manifested by the C- terminus of the molecule.

In this chapter, we report the development of four different monoclonal antibodies to alpha toxin. All four of these antibodies inhibited hemolysis of

rabbit erythrocytes. However, none of these antibodies were directed to the site on the toxin that is responsible for binding to the erythrocyte receptor. As indicated by the IHA test, three of the four antibodies recognized alpha toxin that was already bound to rabbit erythrocytes.

Results

Development of an ELISA for the detection of anti- alpha toxin antibodies.

For the production of monoclonal antibodies, success is crucially dependent on the availability of a quick, reliable and inexpensive assay for specific detection of the antibodies (Goding, 1982). For this reason we developed an ELISA for detection of anti- alpha toxin antibodies. In this assay, the wells of a microtitre plate were coated with alpha toxin and all the remaining binding sites on the plastic were blocked with BSA. Rabbit anti- alpha toxin antibody was then added, followed by goat anti- rabbit enzyme- labelled antibody. Finally, the substrate was added. The assay was modified for detection of monoclonal antibodies by substitution with the hybridoma tissue culture supernatant/ ascites fluid and goat anti- mouse enzyme labelled antibody at the appropriate stages. The controls used in this assay included rabbit pre- immune serum and ascites fluid obtained from pristane- primed BALB/ cbyj mice that were injected with myeloma cells.

The initial experiment was designed to determine the minimal concentration of toxin that resulted in maximal coating of the wells. A serial $\log_2$ dilution of alpha toxin (6 replicates) was added to a washed microtitre plate. After blocking any remaining binding sites on the plastic with BSA, a rabbit anti- alpha toxin preparation diluted 2- fold in PBS was added to all of the wells. Goat anti- rabbit horse radish peroxidase labelled antibody (diluted according to the manufacturer's instructions) was then added. Finally, the substrate o- phenylene diamine (OPD) was added. In the presence of hydrogen peroxide, peroxidase

catalyzes the oxidation of the nearly colourless OPD to a yellowish orange solution which is detected at 490 nm. The results indicate that alpha toxin concentrations of > 200 ng/ well gave maximal well coating (figure 3. 1).

Detection of polyclonal anti– alpha toxin antibody.

Figure 3. 2 shows the detection of rabbit anti– alpha toxin antibody. In this assay the wells of the microtitre plate were coated with toxin at a concentration of 400 ng/ well. The rabbit serum containing anti– alpha toxin antibody was serially diluted $\log_2$ in PBS and then added to the wells (6 replicates). The enzyme labelled antibody was goat anti– rabbit labelled with horse radish peroxidase. The substrate, o–, phenylene diamine (OPD) was oxidized by the peroxidase to yield a yellowish orange solution that was detected at 490 nm.

Detection of monoclonal anti– alpha toxin antibody.

Figure 3. 3 shows the results of a typical experiment to detect monoclonal anti– alpha toxin antibody. In this assay the wells of a microtitre plate were coated with toxin at a concentration of 400 ng/ well. A hybridoma culture supernatant (monoclonal antibody– 3) serially diluted $\log_2$ in PBS– Tween was then added (6 replicates). The enzyme labelled antibody was goat anti– mouse labelled with alkaline phosphatase. The substrate p– nitro phenyl phosphate (PNPP) was hydrolyzed to yield p– nitro phenol (PNP) which was detected at 405 nm. Controls used in this experiment included the supernatant from a myeloma culture that was grown in the same medium as the hybridoma cells.

Monoclonal antibody detection of alpha toxin by SDS PAGE/ immuno electrotransfer blots.

The ability of each monoclonal antibody preparation to recognize alpha toxin was also demonstrated by SDS PAGE/ immuno electrotransfer blotting. Alpha toxin preparations in various stages of purity (*Staphylococcus aureus* culture supernatant, toxin precipitated from the culture supernatant with ammonium sulphate/ methanol, and purified toxin obtained by Sephadex G– 75 chromatography of the ammonium sulphate/ methanol preparation) were electrophoresed on a 10 % SDS PAGE gel. The proteins were then transferred to nitrocellulose membranes and probed with each of the five ascites fluid preparations. Each of the five monoclonal antibodies detected alpha toxin in all of the samples as a single band of molecular weight approximately 34, 000 Daltons. Figure 3. 4 shows a typical densitometric scan of an immunoblot of alpha toxin as detected by monoclonal antibody– 2. The densitometric scans obtained with the other monoclonal antibodies were identical to this figure.

Inhibition of hemolytic activity

In order to determine if the monoclonal antibodies inhibit the hemolytic activity of alpha toxin, each ascites fluid preparation was diluted in PBS and pre– incubated with an equal volume of alpha toxin (50 ul, 1 HU/ ul) for 10 minutes in an ice bath. The mixture was then monitored for hemolytic activity with the Kinetics Hemolysis Assay. All five of the monoclonal antibodies protected the rabbit erythrocytes from toxin– mediated lysis, even when diluted 1: 200. Some ascites fluid preparations protected erythrocytes at even higher dilutions. Figure 3. 5 shows the typical results obtained when different dilutions of the same antibody were used. This assay was performed with monoclonal antibody– 1 diluted 1: 5 (A); 1: 10 (B); 1: 50 (C); 1: 100 (D) and 1: 200 (E). Curve F represents the control which consisted of toxin that was pre– incubated with ascites fluid (diluted 1: 5 in

PBS) obtained from pristane primed BALB/ cbyj mice that were injected with myeloma cells. The results obtained with the other monoclonal antibodies show the same general pattern.

Determination of the relative specificities of the monoclonal antibodies

So far, it has been demonstrated by soluble ELISA, SDS PAGE/ immuno electrotransfer blot and the Kinetics Hemolysis Assay, that all five of the monoclonal antibodies recognize alpha toxin. However, it has not been established whether the antibodies recognize the same, or, different determinants on the toxin molecule. Initially, attempts were made to resolve this question by use of SDS PAGE/ immuno electrotransfer blots, with cyanogen bromide fragments of the toxin. The rationale behind this experiment was, that if all five of the monoclonal antibodies recognize different determinants, using the simplest possible scenario that only one determinant is present on any given fragment, each of the five antibodies should bind to a different fragment. However, despite repeated attempts, the monoclonal antibodies failed to bind to the electroblotted toxin fragments. This phenomenon of monoclonal antibodies binding to blotted antigens with poor specificity, or not at all, has been documented (Bers and Garfin, 1985; Braun *et al.*, 1983; Mandrell and Zollinger, 1984; Steinemann *et al.*, 1984, Turner, 1983). The lack of recognition has been attributed to determinants being denatured by the conditions required for sample preparation, gel electrophoresis and transfer to the nitrocellulose (Bers an Garfin, 1985). Mandrell and Zollinger (1984) suggested that the addition of a zwitterionic detergent such as Zwittergent (Calbiochen– Behring Corp., CA) to the transfer system may restore conformation to the blotted antigen. However, this was also unsuccessful in our system.

To circumvent the problem of denaturation of the determinants, a soluble

ELISA was designed to measure the relative specificities of the monoclonal antibodies. In this assay, the monoclonal antibodies were added singly, and in all of the possible paired combinations to toxin coated wells (400 ng/ well) of a microtitre plate. Goat anti– mouse alkaline phosphatase conjugated antibody was then added followed by the substrate PNPP. Hydrolysis of PNPP to PNP was monitored at 405 nm. The amount of each monoclonal antibody that was individually added to the wells was adjusted to a predetermined concentration (see figure 3. 3) in order to saturate all of the toxin determinants recognized by that particular antibody. Therefore, if two monoclonal antibodies recognize the same determinant, when in combination in the same well, the amount of antibody bound should be the same as when each is added independently. On the other hand, if two antibodies recognize different determinants, when they are both added to the same well, there should be an increase in the amount of antibody bound to the toxin, compared to when each is added individually. Consequently, the amount of bound enzyme– labelled antibody, the amount of substrate hydrolyzed, and the absorbance, should also be proportionately greater. The results of this experiment are given in table 3. 1. From the analysis of these results, it is seen that monoclonal antibodies– 1, 3, 4, and 5 recognize different determinants, while monoclonal antibodies– 1 and 2 recognize the same determinant.

Harshman *et al.*, (1986) reported an anti– alpha toxin monoclonal antibody that recognized the carboxyl terminus of the toxin but did not protect against hemolysis. Using a soluble ELISA similar to the one described above, we compared our monoclonal antibodies to that of Harshman (kindly donated by Dr. Harshman). The results, (table 3. 2) indicate that none of our monoclonal antibodies recognize the same determinant as that of Harshman's. These results are consistent with the fact that all five of our monoclonal antibodies prevent

alpha toxin mediated hemolysis of rabbit erythrocytes, while Harshman's monoclonal antibody does not.

Anti– alpha toxin antibodies that inhibit lysis of erythrocytes; either block the toxin from binding to its receptor on the erythrocyte membrane (antibinding antibodies) or, by neutralizing toxin that is already bound to the receptor (indirect hemagglutinating antibodies) (Lo and Fackrell, 1979). The IHA test (Lo and Fackrell, 1979) was performed on the five monoclonal antibodies, and the results (table 3. 3) indicate that monoclonal antibodies– 1, 2, 4 and 5 agglutinated toxoid–coated erythrocytes. Thus, these monoclonal antibodies were directed to determinants that were outside of the receptor binding site. Monoclonal antibody–3 failed to agglutinate the toxoid coated erythrocytes and thus fell into the category of antibinding antibodies. Antibinding antibodies can be further subdivided into two classes. Class I antibodies recognize a determinant that is in the binding site of the toxin, and thus directly block access to the erythrocyte receptor. Class II antibodies recognize a determinant that is removed from the actual binding site, but, is still close enough so that the bound antibody molecule sterically hinders the toxin from binding to the receptor. Previously, it was demonstrated that a 9, 000 Dalton cyanogen bromide– generated fragment of alpha toxin contains the site that binds to the erythrocyte receptor. In addition, it was also shown that this fragment can be selectively removed by adsorption with erythrocyte ghosts (see chapter 2). A soluble ELISA was then designed in which six wells were coated with unfractionated cyanogen bromide fragments and a duplicate set of wells with cyanogen bromide fragments that were adsorbed with erythrocyte ghosts. If monoclonal antibody– 3 was directed to the binding site it should bind in the first set of wells but not in the second. This should result in a decrease in absorbance in the second set of wells. Table 3. 4 shows that there

was no such reduction in absorbance. Therefore the determinant that is recognized by monoclonal antibody– 3 is outside of the binding site of alpha toxin.

Figure 3. 1

ELISA for detection of anti– alpha toxin antibodies. This assay was designed to determine the lowest concentration of alpha toxin that can be used to obtain maximal coating of the wells of the microtitre plate. The wells (6 replicates) were coated with different concentrations of toxin, and rabbit anti– alpha toxin serum diluted 1: 2 in PBS was added. Goat anti– rabbit antibody labelled with horse radish peroxidase was added next followed by the substrate, o– phenylene diamine (OPD). The substrate was oxidized in the presence of hydrogen peroxide to yield a yellowish orange solution that was detected at 490 nm.

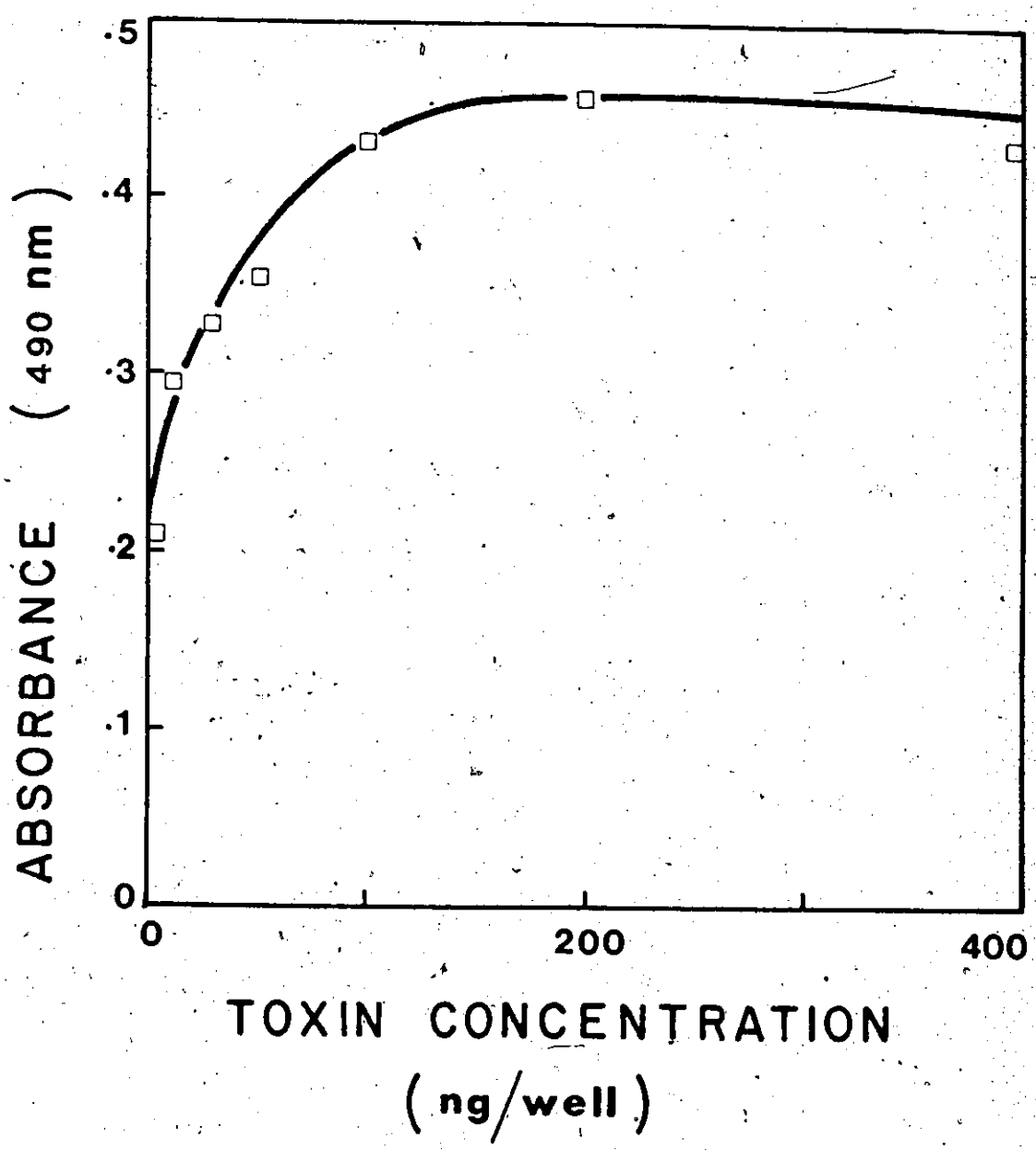

ABSORBANCE (490 nm)
.5
.4
.3
.2
.1
0
0
200
400
TOXIN CONCENTRATION
(ng/well)

Figure 3.2

Detection of rabbit anti– alpha toxin antibody by a soluble ELISA. The wells of the microtitre plate were coated with toxin (400 ng/ well). The anti– alpha toxin rabbit serum serially diluted $\log_2$ in PBS (6 replicates) was added next followed by the horse radish peroxidase labelled goat anti– rabbit antibody. The substrate o– phenylene diamine (OPD) was oxidized to yield a yellowish orange solution that was detected at 490 nm.

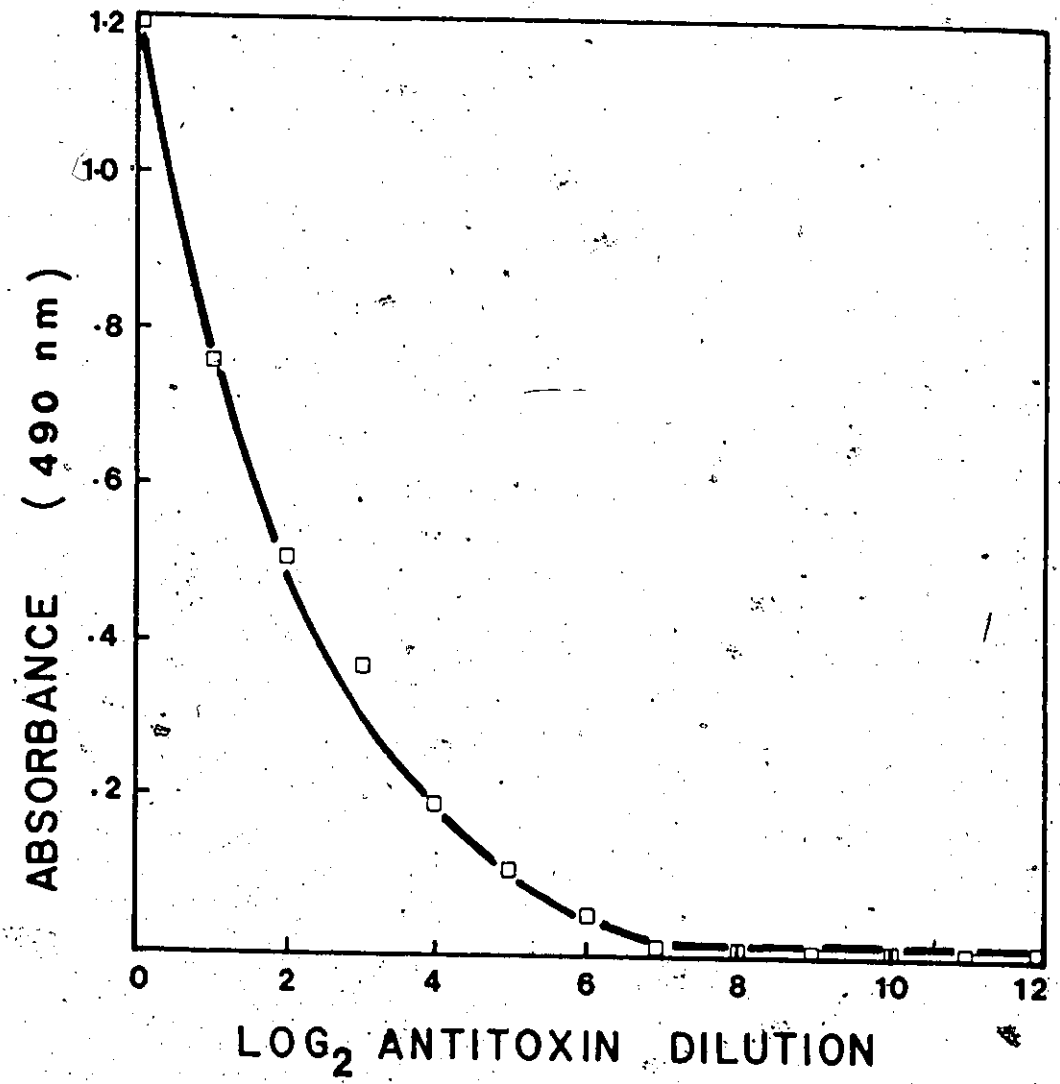

1·2
1·0
·8
·6
·4
·2
ABSORBANCE (490 nm)
0
2
4
6
8
10
12
LOG2 ANTITOXIN DILUTION

Figure 3. 3

Detection of anti- alpha toxin monoclonal antibody- 3 by a soluble ELISA. The wells of the microtitre plate were coated with toxin (400 ng/ well). The hybridoma culture supernatant containing monoclonal antibody- 3, was serially diluted $\log_2$ in PBS and then added to the wells (6 replicates). Goat anti- mouse antibody, labelled with alkaline phosphatase was then added, followed by the substrate p- nitro phenyl phosphate (PNPP). The PNPP was hydrolyzed to p- nitro phenol (PNP) which was detected at 405 nm.

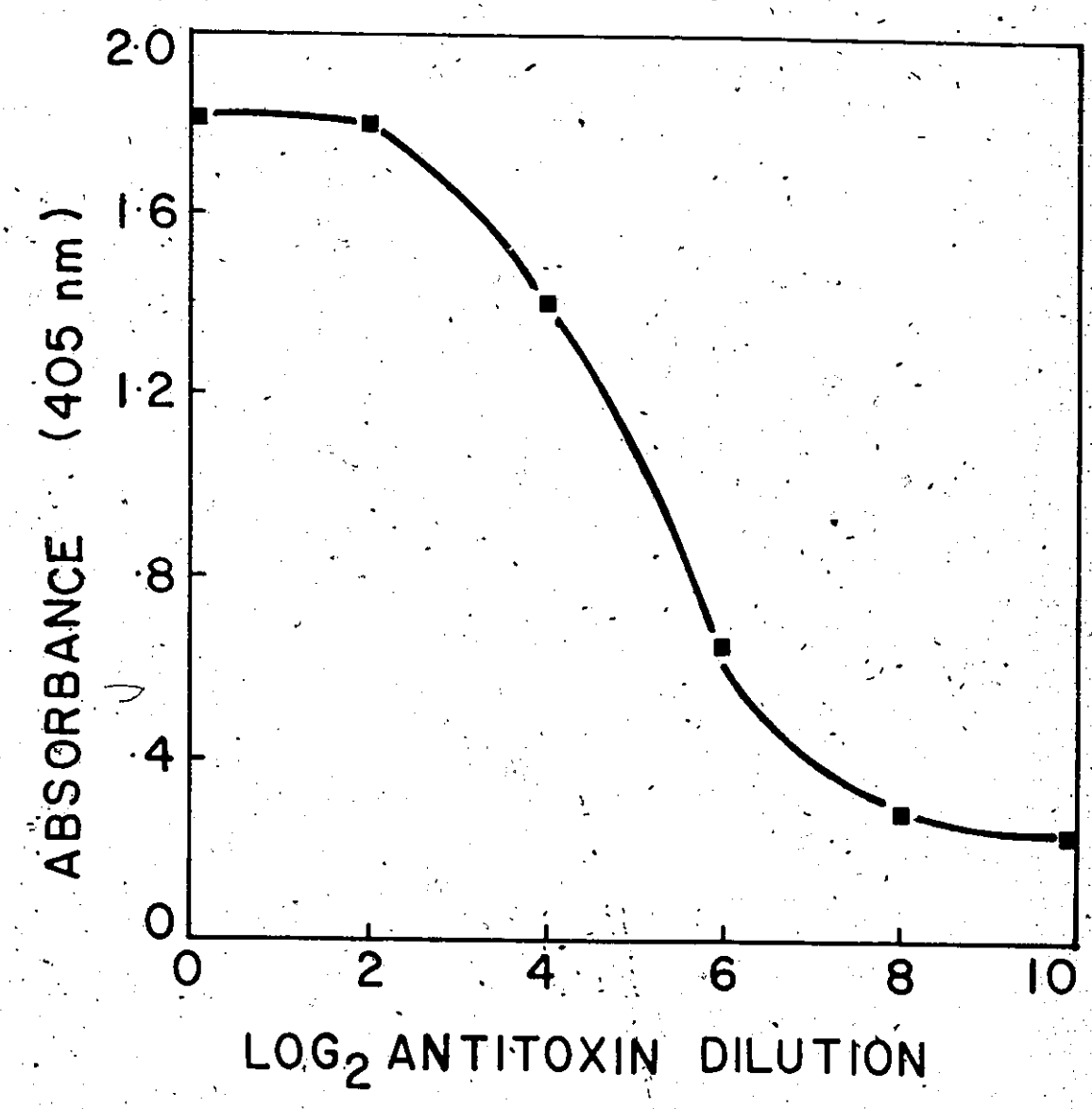

2·0
1·6
1·2
·8
·4
0
ABSORBANCE (405 nm)
0
2
4
6
8
10
LOG2 ANTITOXIN DILUTION

Figure 3. 4

A densitometric scan of an SDS PAGE/ immuno electrotransfer blot of alpha toxin that was detected by anti– alpha toxin monoclonal antibody– 2. Molecular weights are expressed in kiloDaltons. The x– axis represents distance migrated from the top of the gel (cm). The y– axis represents relative absorbance (600 nm). The densitometric scans that were obtained when the other monoclonal antibodies were used, are identical to this figure.

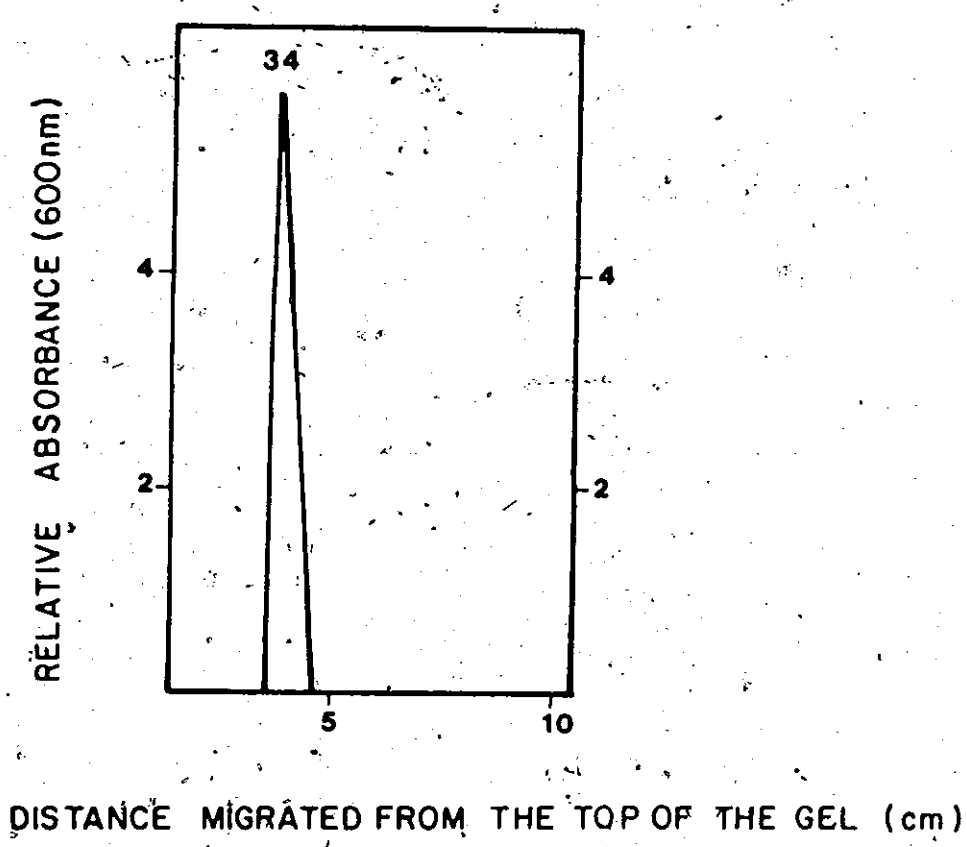

34
4
2
4
2
5
10
RELATIVE ABSORBANCE (600nm)
DISTANCE MIGRATED FROM THE TOP OF THE GEL (cm)

Figure 3. 5

Lysis of rabbit erythrocytes by alpha toxin measured turbidometrically (650 nm) over time. In this assay the toxin was pre– incubated for 10 minutes with different dilutions of anti– alpha toxin monoclonal antibody– 1. The mixtures were then monitored for hemolytic activity by the Kinetics Hemolysis Assay. Curves A to E represent the monoclonal antibody diluted 1: 5; 1: 10; 1: 50; 1: 100; and 1: 200 respectively. Curve F represents the control which consisted of toxin pre– incubated with ascites fluid (diluted 1: 5) obtained from pristane primed BALB/ cbyj mice that were injected with myeloma cells. The results obtained with the other monoclonal antibodies show the same general pattern.

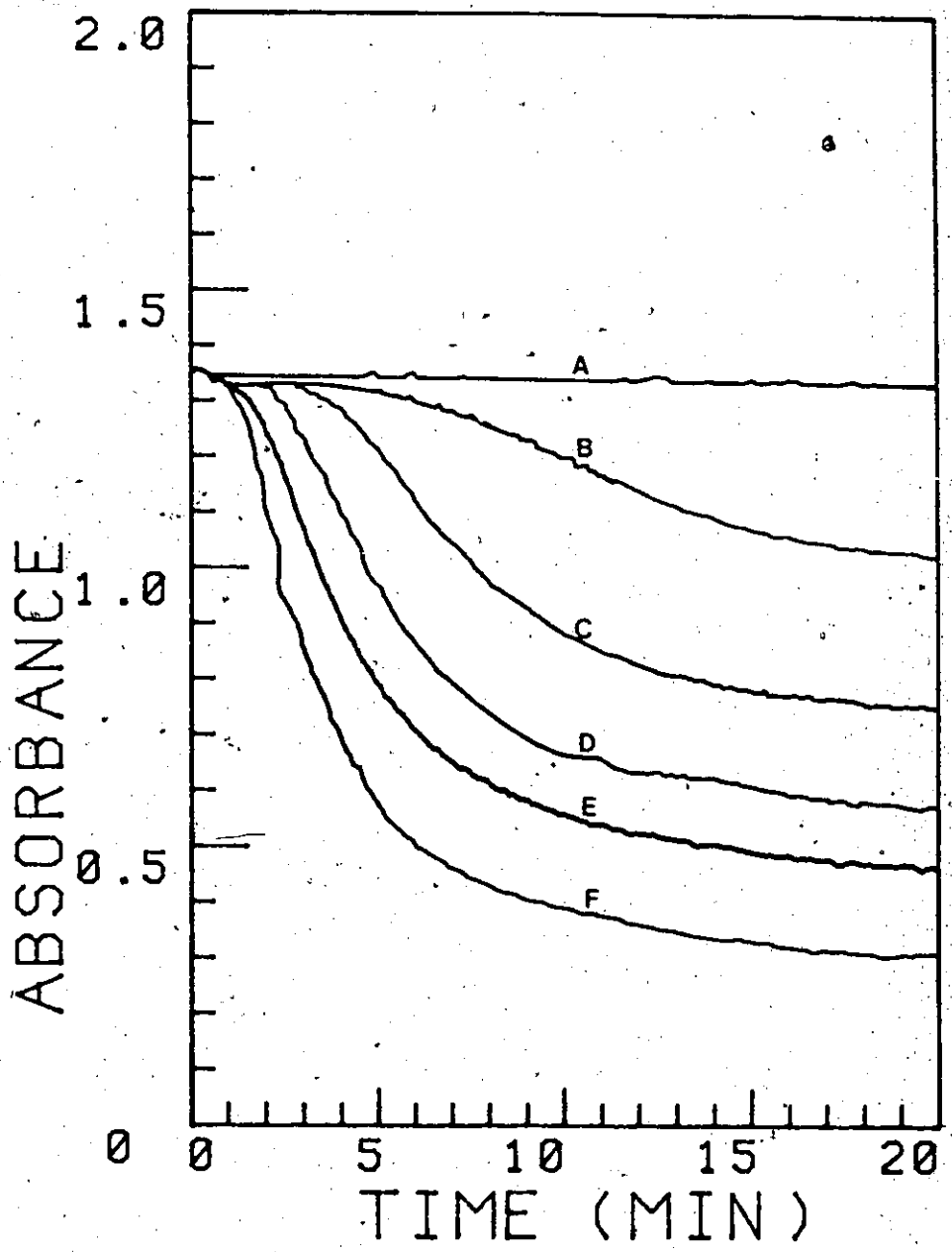

2.0
1.5
1.0
0.5
0
ABSORBANCE
0
5
10
15
20
TIME (MIN)
A
B
C
D
E
F

Table 3. 1

Determination of the relative specificities of the monoclonal antibodies by ELISA

Monoclonal Antibodies	Relative Absorbance (405 nm)
MAb– 1	1.80*+/–(.04)
MAb– 2	1.65+/–(.04)
MAb– 3	0.93+/–(.03)
MAb– 4	1.08+/–(.04)
MAb– 5	1.53+/–(.04)
MAb– 1 + MAb– 2	1.83*+/–(.04)
MAb– 1 + MAb– 3	2.30+/–(.05)
MAb– 1 + MAb– 4	2.24+/–(.04)
MAb– 1 + MAb– 5	2.80+/–(.05)
MAb– 2 + MAb– 3	2.10+/–(.05)
MAb– 2 + MAb– 4	2.12+/–(.04)
MAb– 2 + MAb– 5	2.60+/–(.03)
MAb– 3 + MAb– 4	1.87+/–(.04)
MAb– 3 + MAb– 5	2.22+/–(.04)
MAb– 4 + MAb– 5	2.36+/–(.04)

The wells of the microtitre plate were coated with alpha toxin (400 ng/ well). The concentrations of the monoclonal antibodies that were added individually to the wells (6 wells/ MAb) were adjusted so that all the determinants recognized by that particular antibody were saturated. Any increase in absorbance that was noted when the antibodies were added in pairs was therefore an indication that two separate determinants were recognized in that well. Those two antibodies were therefore specific for different determinants. On the other hand, if the addition of two antibodies to the same well did not result in an increase in absorbance, then those two antibodies probably recognized the same determinant. This experiment was performed a total of three times. The results obtained on each occasion showed the same pattern. The absorbance values given in this table are the arithmetic means and standard errors ( ) from one such experiment. *– denotes not significantly different as determined by the F test.

Table 3. 2

A comparison of the relative specificities of MAb– 1, 2, 3, 4 and 5 with that of MAb– A–Tox–653.1.

Monoclonal Antibody	Relative Absorbance (405 nm)
MAb– 1	0.77+/–(.04)
MAb– 2	0.95+/–(.03)
MAb– 3	0.79+/–(.04)
MAb– 4	0.42+/–(.03)
MAb– 5	0.78+/–(.03)
MAb– A–Tox–653.1	0.78+/–(.04)
MAb– 1 + MAb– A–Tox–653.1	0.99+/–(.04)
MAb– 2 + MAb– A–Tox–653.1	1.27+/–(.04)
MAb– 3 + MAb– A–Tox–653.1	0.99+/–(.03)
MAb– 4 + MAb– A–Tox–653.1	1.24+/–(.04)
MAb– 5 + MAb– A–Tox–653.1	1.37+/–(.04)

The wells of the microtitre plate were coated with alpha toxin (400 ng/ well). The concentrations of the monoclonal antibodies that were added individually to the wells (6 wells/ MAb) were adjusted so that all the determinants recognized by that particular antibody uere saturated. Any increase in absorbance that was noted when any antibody was added together with MAb– A–Tox–653.1, was therefore an indication that two separate determinants were recognized in that well. Those two antibodies were therefore specific for different determinants. This experiment was performed a total of three times with the results showing the same pattern on each occasion. The absorbance values expressed in this table are the arithmetic means and standard errors ( ) from one experiment. Mab– A– Tox–653.1 was kindly donated by Dr. S. Harshman.

Table 3. 3

Results of an IHA test performed on MAb– 1, 2, 3, 4 and 5.

Monoclonal Antibody	IHA Titre
MAb– 1	256
MAb– 2	256
MAb– 3	4
MAb– 4	128
MAb– 5	512

The IHA test was performed as described in Materials and Methods, a total of three times using different batches of each ascites fluid. The results that were obtained on each occasion showed the same pattern. The titres displayed in this table represent the results of one such experiment.

Table 3. 4

Results of a soluble ELISA that was designed to determine if MAb– 3 recognized the erythrocyte receptor binding site of alpha toxin.

Test Antigen	Relative Absorbance (405 nm)
CNBr fragments	1.65+/–(.04)
CNBr fragments that were adsorbed with rabbit erythrocyte ghosts	1.71+/–(.04)

In this assay six wells of a microtitre plate were coated with an unfractionated population of cyanogen bromide generated fragments of alpha toxin. A duplicate set of wells was coated with rabbit erythrocyte ghosts– adsorbed cyanogen bromide fragments. This adsorption procedure removed the fragment that contained the erythrocyte membrane receptor binding site of alpha toxin. A reduction in titre in the second set of wells would indicate that the antibody recognized a determinant on the fragment that was adsorbed out. This assay was performed a total of four times. The results obtained on each occasion showed the same degree of difference between the two samples. The results displayed in this table are the arithmetic means and standard errors ( ) from a single experiment.

Discussion

Five monoclonal antibodies (designated monoclonal antibody–1 to 5) were produced from hybridoma cells that were prepared from fusions of spleen cells obtained from BALB/ cbyj mice, and P3X63– Ag8.653 myeloma cells. Heat inactivated alpha toxin was used as the immunogen, and both *in vitro*, and *in vivo* immunization techniques were used. The hybridoma cells that were produced, were screened by a soluble ELISA for production of anti– alpha toxin antibodies. Some of the antibody– producing hybrids were cloned by limiting dilution and then used to produce ascites fluid in mice that were previuosly primed with pristane. Hybridoma cells that were produced in the ascites fluid were sedimented by centrifugation and reinjected into pristane– primed mice for a total of three passages.

Four of the monoclonal antibodies produced (monoclonal antibody– 1, 3, 4, and 5) were shown by a soluble ELISA to be directed to independent determinants. Monoclonal antibodies– 1 and 2 seem to recognize the same determinant. However, the possibility that these two antibodies are directed to determinants that are separate but physically close together, cannot be disregarded. It has been shown that in such situations, the antibodies may fail to bind to the antigen simultaneously (Atassi, 1967; 1975). So far, there has been only one other report of a monoclonal antibody produced against alpha toxin (Harshman *et al.*, 1986). This monoclonal antibody has been shown to recognize a determinant in the C terminus region of the toxin, but does not protect against alpha toxin mediated hemolysis. A comparison of this antibody with each of ours, has indicated that none of our antibodies recognize this C terminus determinant.

All five of the monoclonal antibodies protected rabbit erythrocytes from alpha toxin mediated lysis. Using the IHA test, monoclonal antibodies- 1, 2, 4, and 5 were shown to recognize toxoid that was bound to erythrocyte membranes. These were thus classified as IHA antibodies. Monoclonal antibody- 3 failed to agglutinate the toxoid- coated erythrocytes and was therefore classified as an antibinding antibody. Alpha toxin mediated hemolysis of rabbit erythrocytes is currently believed to involve the following sequence of events: (1) binding of the toxin monomers to specific receptors on the membrane (2) a conformational change in the toxin followed by the formation of a hexameric complex which is thought to penetrate the lipid bilayer to form a channel (3) leakage of small ions through the channel and (4) colloid osmotic lysis of the cell (Bhakdi *et al.*, 1984; Blomqvist and Thelestam, 1986b; Cassidy and Harshman, 1976b; Cassidy and Harshman, 1979; Freer and Arbuthnott 1983; Harshman *et al.*, 1986). Since the IHA antibodies neutralize toxin that is already bound to the receptor, they probably function by preventing the conformational change, or, by preventing the hexamer formation. The antibinding antibodies function by either of two ways. They can directly block the binding site of the toxin and thus inhibit receptor recognition. Alternatively, they may recognize a determinant which is outside of the actual binding site, but, is still close enough so that simultaneous binding to the receptor is precluded by steric hindrance. Monoclonal antibody- 3 has been shown to belong to this latter class due to its failure to react with the cyanogen bromide fragment of the toxin that contains the binding site.

In an SDS PAGE/ immuno electrotransfer blot system, each of the five monoclonal antibodies recognized alpha toxin in various stages of purity, as a single band of molecular weight approximately 34, 000 Daltons. However, in the same system all of the antibodies failed to recognize fragments of the toxin. The

failure of monoclonal antibodies to react with blotted antigens has been documented (Bers and Garfin, 1985; Braun *et al*, 1983; Mandrell and Zollinger, 1984; Steinemann *et al*, 1984; Turner, 1983). This phenomenon has been attributed to denaturation of the determinants during sample preparation, gel electrophoresis or transfer to the nitrocellulose membrane (Bers and Garfin, 1985). Even the addition of a zwitterionic detergent to the transfer system, as suggested by Mandrell and Zollinger (1984) failed to restore conformation to the blotted fragments. On the other hand, the monoclonal antibodies were seen to react with the toxin fragments in a soluble ELISA (data not shown). Since the ELISA does not employ the relatively harsh conditions that are required in SDS PAGE/ immuno electrotransfer blotting, in this assay, the determinants probably retained their native conformations.

Further studies with the monoclonal antibodies to determine whether they inhibit the other biological activities of alpha toxin are now in progress. These experiments coupled with fragmentation analyses and amino acid sequence studies of the different fragments should allow a detailed structure– function mapping of alpha toxin.

OVERVIEW

The three main objectives of this study, the development of an alternative assay for detection and measurement of alpha toxin, fragmentation analysis of alpha toxin, and the production of monoclonal antibodies to alpha toxin, were all accomplished.

A double antibody sandwich ELISA was developed for the detection of alpha toxin. This assay overcomes several problems that are inherent in the hemolytic titration assay. These include, a lack of standardization of the hemolytic unit, and the high degree of variability often seen in the hemolytic titration assay. In addition, biologically inactive alpha toxoid, which is always found in preparations of alpha toxin, is also measured by the ELISA. Finally, the ELISA was found to be 500- 1, 000 times more sensitive than the hemolytic titration assay. One possible inconvenience with this ELISA is the presence of protein A which may also be produced by some *Staphylococcus aureus* strains. However, the protein A from such culture fluids can be depleted by adsorption with IgG coated beads.

A modified version of the double antibody sandwich ELISA, a competitive enzyme linked immunosorbent assay (CELIA) was then developed for the detection of immunologically reactive fragments of alpha toxin. In the CELIA, immunologically reactive fragments bound to the adsorbed anti- alpha toxin antibodies, and thus precluded these antibodies from binding subsequently added intact alpha toxin. Since these fragments were unable to simultaneously bind the second (enzyme- labelled) antibody, their presence was indicated by a decrease in the absorbance in these wells.

The third immunoassay developed was an ELISA for the detection of both rabbit polyclonal antisera and monoclonal antisera. In this assay, the microtitre plate was coated with alpha toxin. The anti- alpha toxin antibodies

were next added, followed by the enzyme- labelled indicator antibody, and then the substrate. It was found that an alpha toxin concentration of >200 ng/ well gave maximal well coating. This assay was routinely used for screening hybridomas for the presence of antitoxin- producing hybrid cells.

Finally, an SDS PAGE/ immuno electrotransfer blot system was developed for the detection of intact alpha toxin and its immunologically reactive fragments. In this assay, the proteins that were separated by SDS PAGE, were transferred to nitrocellulose and probed with either the polyclonal or monoclonal antibodies. Fragmentation analysis of alpha toxin with trypsin, produced a variety of fragments some of which reacted with antibinding antibody and others with indirect hemagglutinating antibody. However, the most common and striking result of these experiments was a fragment of approximately 20, 000 Daltons. This fragment which appeared early in the digestion course and was resistant to further tryptic digestion, reacted with both populations of antibody. A similar sized fragment was also detected in preparations of native alpha toxin by SDS PAGE/ immuno electrotransfer blotting. Blomqvist and Thelestam (1986a) also reported a similar fragment that spontaneously appeared in their alpha toxin preparations. This fragment contained the sites responsible for binding to the mouse target receptor, and for hemolysis of rabbit erythrocytes. However, since the biological properties of our fragment are not known, comparisons with the fragment reported by Blomqvist and Thelestam (1986a) are not yet possible.

Cyanogen bromide digestion of alpha toxin produced five fragments that were recognized by polyclonal antitoxin. The apparent molecular weights of these fragments ranged from 9, 000 to 12, 000 Daltons. The 9, 000; 10, 000 and 12, 000 Dalton fragments reacted with antibinding antibodies. The 9, 000; 10, 000; 10, 500 and 11, 000 Dalton fragments reacted with IHA antibodies. The 9, 000 Dalton

fragment was also shown to contain the site responsible for binding to the rabbit erythrocyte receptor. Lo (1984, PhD Dissertation) reported that alpha toxin contains one IHA determinant. The multiplicity of IHA fragments that are reported here may be explained by any of four possible reasons. Firstly, Lo (1984, PhD Dissertation) measured the number of epitipes on alpha toxin. The toxin used in this study was fragmented, thus cryptotopes were also accounted for. Secondly, different antisera may recognize different antigenic determinants. (Atassi, 1975; Atassi and Saplin, 1968). Thirdly, some determinants may be so close together that binding of one antibody would sterically preclude another antibody from binding to an adjacent determinant (Atassi, 1967; Atassi, 1975). This constraint was lifted when the protein was fragmented. Finally, alpha toxin has been shown to undergo a change in tertiary structure upon binding to its membrane receptor (Tobkes *et al.*, 1985). Since the IHA antibodies can only be isolated from toxoid already bound to erythrocytes, it is possible that IHA determinants are only exposed after the toxin becomes bound to its receptor, and undergoes the conformational change. These determinants would not be available on the native toxin in solution, that was used by Lo (1984, PhD Dissertation).

Five monoclonal antibodies (MAb-1 to MAb-5) to alpha toxin were developed. However, when the relative specificities of these antibodies were examined by ELISA, MAB-1 and MAB-2 were found to recognize the same determinant, or, two determinants that are physically close together. Monoclonal antibodies 1, 3, 4, and 5 recognized different determinants. All the monoclonal antibodies protected rabbit erythrocytes from alpha toxin- mediated lysis. When examined in the IHA test, MAb-1, 2, 4, and 5 agglutinated toxoid- coated erythrocytes. This indicated that these antibodies recognize determinants that are outside of the erythrocyte receptor binding site of the toxin. Monoclonal antibody- 3, an antibinding

antibody, did not recognize the 9, 000 Dalton CNBr fragment that contains this binding site. Monoclonal antibody- 3 therefore recognizes a determinant that is outside of the binding site, but, is still close enough so the simultaneous binding to the receptor is precluded.

Continuation of this project should first concentrate on isolation of the tryptic and CNBr fragments of alpha toxin. Attempts at isolation were made with a reverse phase HPLC system which utilized acetonitrile (+ 0.1 % (V/ V) trifluoroacetic acid) as the mobile phase. However, it was found that alpha toxin and its fragments precipitated in this system. It is suggested that future attempts at HPLC separation utilize a gel filtration system with physiological buffers. Once the fragments are isolated, their biological properties, such as hemolytic, dermonecrotic, and lethal activities should be investigated. This combined with amino acid analysis of the fragments should result in the evolution of a structure-function map such as that suggested by Blomqvist and Thelestam (1986a).

The SDS PAGE/ immuno electrotransfer blot system has many potential applications, some of which have already been initiated in this laboratory. Polyclonal and monoclonal anti- band 3 and anti- alpha toxin antibodies have been used as probes in this system. This has led to the identification of alpha toxin receptors in various tissues. The isolation, characterization and comparison of these receptors is now in progress.

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Academic Awards

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1977 – 1981	President's Roll, University of Windsor
1982 – 1985	Summer Bursary, University of Windsor
1981 – present	Summer Research Assistantship, Biology Department, University of Windsor
1981 – present	Teaching Assistantship, Biology Department, University of Windsor

University Service

1983 – 1985 Elected graduate student representative to the Graduate Committee, Biology Department, University of Windsor.

Experience

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Publications

1. Fackrell, H.B., O.P. Surujballi, S.R. Burgess. 1985. **ELISA Data Reduction: A Review**. J. Clin. Immunoassay. 8:213–219

2. Surujballi, O.P., and H.B. Fackrell. 1984. **Enzyme– Linked Immunosorbent Assay for *Staphylococcus aureus* alpha toxin.** J. Clin. Microbiol. 19:394–398.

Papers Presented at Professional Meetings

1. Surujballi, O.P., and H.B. Fackrell. 1983. **ELISA for Staphylococcal alpha toxin**. Joint Fall Meeting, Michigan Branch of The American Society for Microbiology and Central Ontario Branch of The Canadian Society for Microbiology. Windsor, Ontario.

2. Surujballi, O.P., and H.B. Fackrell. 1985. **Antigenic fragments of staphylococcal alpha toxin**. Annual Meeting, Canadian Congress of Biology. London, Ontario.

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